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13. ABSTRACT *(Maximum 200 words)*

Defects in repair of double strand chromosome breaks (DSBs) are emerging as important factors both in familial and sporadic breast tumors. Just as a bacterial model for single-base mismatch repair lead to rapid identification of human homologues with roles in carcinogeneis, development of a bacterial model for DNA DSB repair will provide important insights into the role this system plays in breast tumor development. Our work toward development of a model for bacterial DSB repair by replication coupled to recombination has progressed significantly during the course of this grant contract period. We determined that the *Escherichia coli* PriA protein is absolutely required for coupling DNA replication with recombination both *in vivo* and *in vitro*. PriA specifically recognized a replication origin created by recombination and showed a preference for forked DNA structures in gel shift assays. PriA's 3' to 5' helicase activity unwound the DNA at a fork and promoted loading of the replicative helicase DnaB, a central step in assembly of the replisome. These data indicate that PriA is an initiator protein that responds to DNA damage and assembles the components necessary for repair by replication, maintaining the integrity of the chromosome and preventing potentially catastrophic loss of information and mutation.

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FOREWORD

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PI - Signature

6/10/99
Date

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Introduction:

Genetic defects in breast tumors frequently involve mutations in both oncogenes and tumor suppressor genes. Genes involved in the repair of DNA can be classified as tumor suppressor genes, but thus far only genes required for one type of DNA repair, single-base mismatch repair, have been fully characterized in humans. While defects in these genes appear to play a role in a small number of breast tumors, defects in repair of double strand chromosome breaks (DSBs) are emerging as important factors both in familial and sporadic breast tumors. We have focussed on development of a bacterial model for repair of DSBs by replication coupled to homologous recombination, and such a system will likely provide insight into the mechanism of DSB repair in humans. The reconstituted system for bacteriophage Mu replication by transposition has been an invaluable tool in this process. During Mu transposition, strand exchange catalyzed by the phage-encoded transposase MuA leads to formation of a branched DNA structure with a potential replication fork at either end of the transposing DNA element, similar to the branched intermediates created during homologous recombination. Bacterial proteins including the replicative helicase DnaB and DNA polymerase III holoenzyme then assemble a replisome at one end this substrate and commence semi-discontinuous DNA synthesis from one end to the other. Like replication coupled to recombination on the bacterial chromosome, initiation of bacteriophage Mu replication is independent of the chromosomal initiator protein DnaA, suggesting that bacteriophage Mu may harness the cellular apparatus required for coupling replication with recombination. Our finding that the *Escherichia coli* PriA protein was required for Mu replication by transposition both *in vivo* and *in vitro* supported this hypothesis. Previous to our work, PriA had been hypothesized to couple replication with homologous recombination based on genetic evidence and on the role of PriA in assembly of a primosome for bacteriophage ϕX174 complementary strand synthesis. Our work provided the first definitive biochemical evidence that PriA could couple replication with recombination.

Report Body

Summary of Progress:

When this grant was first reviewed, our initial findings regarding the role of PriA in Mu DNA replication were under submission for publication. They have since been published (manuscript reprint attached). We proposed to expand upon these finding with three Specific Aims:

1) Identification of additional *E. coli* proteins required for Mu DNA replication.
2) Analysis of Mu DNA sequences and structures promoting assembly of the primosome.
3) Development of a reconstituted system for identifying proteins required for DSB-induced replication

During the first year of this grant, significant progress has been made on Specific Aim #2, with all tasks in the approved Statement of Work being completed. These findings have also been published (manuscript reprint attached). Our work on Specific Aim #2 revealed the importance of the helicase activity of PriA for the initiation of Mu DNA replication, and we have expanded our exploration of this activity. A manuscript is in preparation describing factors that influence initiation of helicase activity, although this work is not yet complete. We have also completed Tasks #1 and #4 of Specific Aim #1. Task #1 was purification of *E. coli* preprimosome components PriB, PriC and DnaT, and all three have now been purified to 95% or greater homogeneity. Task #4 was examination of the ability of a *ruvA*$^-$ strains to support Mu lytic development (i.e. replication by transposition) *in vivo*. We found that *ruvA*$^-$ strains support Mu lytic growth, as do *ruvC*$^-$, *recG*$^-$, *recF*$^-$, *recO*$^-$, and *recR*$^-$ strains. Summaries of our published and unpublished work to are provided below.

The principal investigator on this grant, pre-doctoral candidate Jessica M. Jones, received her degree on April 30, 1999, and has moved to the laboratory of Dr. Martin Gellert at the NIH/NIDDK where she is studying the biochemical mechanism of DSB repair in mammalian cells. In addition to being awarded University Honors on her thesis work supported in part by this grant, Dr. Jones was also chosen as the 1999 recipient of the Nat Sternberg Thesis Award, an international award given for outstanding pre-doctoral work in the field of prokaryotic molecular biology. This grant also contributed to Dr. Jones' professional development through travel to several scientific meetings, including the 1999 Keystone Symposium on Replication and Recombination where Dr. Jones was a workshop speaker. Dr. Jones's pre-doctoral mentor, Dr. Hiroshi Nakai, is currently recruiting another candidate to complete Specific Aims #1 and #3 of this grant.

Research Summary:

<u>The ϕX174-type primosome promotes replisome assembly at the site of recombination in bacteriophage Mu transposition</u>; J. M. Jones and H. Nakai (1997), EMBO J., *16*:6886-6895.

Initiation of *Escherichia coli* DNA synthesis primed by homologous recombination is believed to require the ϕX174-type primosome, a mobile priming apparatus assembled without the initiator protein DnaA. We show that this primosome plays an essential role in bacteriophage Mu DNA replication by transposition. Upon promoting transfer of the Mu ends to target DNA, the Mu transpososome undergoes transition to a prereplisome that

permits initiation of DNA synthesis only in the presence of primosome assembly proteins PriA, DnaT, DnaB and DnaC. These assembly proteins promote the engagement of primase and DNA polymerase III holoenzyme, initiating semi-discontinuous replication preferentially at the left end of Mu. The results indicate that these proteins play a crucial role in promoting replisome assembly on a recombination intermediate.

Duplex opening by primosome protein PriA for replisome assembly on a recombination intermediate; J. M. Jones and H. Nakai (1999), J. Mol. Biol., *289*:503-515.

PriA and other primosome assembly proteins of *Escherichia coli* recruit the major replicative helicase DnaB for replisome assembly during bacteriophage Mu transposition and replication. MuA transposase catalyzes the transfer of Mu ends to target DNA, forming a potential replication fork that provides the assembly site for the replisome. However, this fork lacks the single-stranded DNA needed to load DnaB. Although no pre-existing primosome assembly sites that bind PriA were found within the Mu end sequences, PriA was able to bind the forked DNA structure created by MuA. The helicase activity of PriA could then open the duplex to create the DnaB binding site. In a tightly coupled reaction on synthetic fork substrates, PriA promoted both the unwinding of the lagging strand arm and preprimosome assembly to load DnaB onto the lagging strand template. PriA apparantly translocated 3' to 5' along the lagging strand template until sufficient single-stranded DNA was exposed for binding of DnaB, which then translocated 5' to 3' in the opposite direction. Mutant PriA lacking helicase activity was unable to promote this process, and loss of PriA helicase impaired Mu DNA replication *in vivo* and *in vitro*. This suggests that the opening of the duplex by PriA is a critical step in the initiation of Mu DNA replication. Concerted helicase and primosome assembly functions would allow PriA to act as an initiator on recombination intermediates and stalled replication forks. As part of the replisome, PriA may act as a mobile initiator that minimizes interruptions in chromosomal replication.

The PriA helicase of *Escherichia coli*: Effect of DNA structure and additional proteins on helicase activity; J. M. Jones and H. Nakai, *manuscript in preparation.*

The PriA primosome assembly protein of *Escherichia coli* couples DNA recombination with replication and is hypothesized to assist in replication restart following replication fork collapse. The 3' to 5' helicase activity of PriA can assist in primosome assembly by opening the duplex at a DNA fork to create a binding site for the replicative helicase DnaB. PriA helicase was most active on forked DNA substrates with structures similar to a collapsed replication fork and required a small (2 base or larger) single-stranded gap at the fork to initiate unwinding. Helicase activity was self-limiting, with PriA efficiently unwinding duplexes as long as 300 bp but not longer. Additional primosome components PriB, PriC and DnaT had no effect on PriA helicase activity at a fork, but they could inhibit PriA translocation on single-stranded DNA coated with single-strand binding protein (SSB). SSB inhibited PriA helicase activity on forked substrates where PriA and SSB bound to the same strand, although SSB did not inhibit PriA binding. SSB, PriBC/DnaT and fork structure together may ensure that PriA helicase activity is confined to substrates such as collapsed replication forks that lack sufficient single-stranded DNA for preprimosome assembly.

Key Research Findings

• The *Escherichia coli* PriA protein couples replication with recombination.

• PriA is absolutely required for bacteriophage Mu replication by transposition *in vivo* and *in vitro*.

• PriA recognizes the forked DNA intermediate created by strand exchange during homologous or non-homologous recombination or by replication fork collapse. This recognition is independent of sequence.

• The helicase activity of PriA contributes significantly to bacteriophage Mu replication by transposition.

• The helicase and primosome assembly activities of PriA can be coupled, allowing PriA to catalyze primosome assembly on forked intermediates that would otherwise lack sufficient single-stranded DNA.

• The helicase activity of PriA is confined primarily to substrates where insufficient single-stranded DNA is available for primosome assembly, such as collapsed replication fork structures, and is less active on substrates where ample single-stranded DNA is available, such as D-loop homologous recombination intermediates.

Reportable Outcomes

Manuscripts:

The ϕX174-type primosome promotes replisome assembly at the site of recombination in bacteriophage Mu transposition. J. M. Jones and H. Nakai (1997), EMBO J., *16*:6886-6895.

Duplex opening by primosome protein PriA for replisome assembly on a recombination intermediate. J. M. Jones and H. Nakai (1999), J. Mol. Biol., *289*:503-515.

The PriA helicase of *Escherichia coli*: Effect of DNA structure and additional proteins on helicase activity. J. M. Jones and H. Nakai, *manuscript in preparation.*

Degrees Obtained:

Doctor of Philosophy in Biochemistry and Molecular Biology conferred upon Jessica M. Jones, April 30, 1999

Employment Received:

IRTA Fellowship from the NIH/NIDDK awarded to Dr. Jessica M. Jones. Dr. Jones will perform research in the area of mammalian DSB repair in the laboratory of Dr. Martin Gellert.

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The φX174-type primosome promotes replisome assembly at the site of recombination in bacteriophage Mu transposition

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Initiation of *Escherichia coli* DNA synthesis primed by homologous recombination is believed to require the φX174-type primosome, a mobile priming apparatus assembled without the initiator protein DnaA. We show that this primosome plays an essential role in bacteriophage Mu DNA replication by transposition. Upon promoting transfer of Mu ends to target DNA, the Mu transpososome undergoes transition to a pre-replisome that permits initiation of DNA synthesis only in the presence of primosome assembly proteins PriA, DnaT, DnaB and DnaC. These assembly proteins promote the engagement of primase and DNA polymerase III holoenzyme, initiating semi-discontinuous replication preferentially at the Mu left end. The results indicate that these proteins play a crucial role in promoting replisome assembly on a recombination intermediate.

Keywords: *in vitro* DNA replication/phage Mu/primosome/replisome/transposition

Introduction

Coupling of DNA synthesis to recombination is an important mechanism involved in DNA repair, genetic exchange and chromosomal replication. Growing evidence suggests interdependence between chromosomal replication and homologous recombination, DNA replication participating in the formation of recombinants and homologous recombination leading to initiation of chromosomal replication (Kogoma *et al.*, 1996). Involvement of the primosome assembly protein PriA in both recombinant formation and recombination-dependent DNA replication in *Escherichia coli* has suggested that it may be part of an apparatus for linking strand exchange with DNA synthesis.

PriA is a constituent of the φX174-type primosome, which originally was characterized for its function in converting single-stranded phage φX174 DNA to the duplex replicative form (Kornberg and Baker, 1992). It is distinguished from the *oriC*-type primosome by the involvement of host-encoded PriA, PriB, PriC and DnaT proteins in primosome assembly instead of the initiator protein DnaA, which promotes replisome assembly at the bacterial origin of replication. In φX174 replication, PriA binds to the unique primosome assembly site (PAS) on single-stranded phage DNA and recruits PriB, PriC and DnaT (Shlomai and Kornberg, 1980; Liu *et al.*, 1996; Ng and Marians, 1996a). With the assistance of the associated matchmaker DnaC, DnaB helicase is then delivered to the complex to form the preprimosome. DnaB within this mobile apparatus interacts transiently with primase to form the primosome (Tougo *et al.*, 1994; Ng and Marians, 1996b), which catalyzes synthesis of RNA primers at many sites on the template to initiate DNA synthesis by the DNA polymerase (pol) III holoenzyme (Ng and Marians, 1996b).

PriA's ability to promote primosome assembly plays an important role in DnaA-independent DNA synthesis such as pBR322 replication (Minden and Marians, 1985). On a preformed replication fork, which is a circular duplex with a single-stranded tail, PriA can promote the assembly of a replisome that catalyzes leading and lagging strand synthesis if a PAS is present on the tail (Wu *et al.*, 1992). However, the φX-type primosome is not necessarily required for replication of the bacterial chromosome. DNA replication initiated at *oriC* can be reconstituted *in vitro* without the PriA, PriB, PriC and DnaT proteins (Kaguni and Kornberg, 1984). Strains with *priA* null mutations are viable although they display characteristics of slow growth, filamentous structure, increased sensitivity to DNA-damaging agents and a constantly induced SOS system (Lee and Kornberg, 1991; Nurse *et al.*, 1991). It has been suggested that the φX-type primosome may be required for reinitiation should the replisome stall (Nurse *et al.*, 1991). Recent evidence demonstrates that *priA* null strains show poor assimilation of genetic markers by homologous recombination and are defective in DNA double strand break repair (Kogoma *et al.*, 1996). They are also deficient in inducible and constitutive stable DNA replication (iSDR and cSDR) (Masai *et al.*, 1994), forms of chromosomal replication which occur independently of the DnaA protein.

Since iSDR is dependent on homologous recombination functions, a model has been proposed for the function of the φX-type primosome in coupling recombination with replication (Asai and Kogoma, 1994; Kogoma, 1996). The potential replication fork is produced when an invading strand displaces one strand of a duplex to form a D-loop structure (Eggleston and West, 1996) and provides the potential primer for leading strand synthesis. The φX-type primosome is assembled on the single-stranded region within the D-loop, promoting replisome assembly and establishing a replication fork (Kogoma, 1996). In support of this hypothesis, DnaT and DnaC, which are also involved in the assembly of the φX-type primosome, are required for iSDR as well (Masai and Arai, 1988). In addition, PriA can bind to D-loops and related DNA structures (McGlynn *et al.*, 1997). However, the ability of the φX-type primosome to promote initiation of replication on a natural recombination intermediate has heretofore not been demonstrated.

Phage Mu DNA replication by transposition resembles the hypothesized mechanisms for DNA replication coupled

Table I. PriA⁻ *Escherichia coli* hosts can support Mu lysogenization but not lytic development

Host strain[a]	Relevant trait	Mu plating efficiency	Frequency of lysogenization
EL501	PriA⁺	1	8×10^{-3}
EL500	PriA⁻	$<10^{-7}$	0.7×10^{-3}
EL502	PriA⁺	0.8	not determined
AT3327	PriA⁺	1	4×10^{-3}
AT3327 *priA1::kan*	PriA⁻	$<10^{-7}$	0.8×10^{-3}

[a]EL501 and EL500 are an isogenic pair; EL500 contains a 1.3 kb insertion in the *priA* gene (*priA1::kan*) (Lee and Kornberg, 1991). EL502 also contains this insertion but has been transformed with plasmid pEL042 expressing PriA (Lee *et al.*, 1990).

to homologous recombination. In Mu transposition, strand exchange is catalyzed by the phage-encoded transposase MuA (for reviews, see Mizuuchi, 1992; Chaconas *et al.*, 1996; Lavoie and Chaconas, 1996). Monomeric MuA binds to specific sequences at each Mu end (Craigie *et al.*, 1984; Kuo *et al.*, 1991), assembling into a tetramer that holds together the two ends (Lavoie *et al.*, 1991). This transpososome introduces a nick at each end, and the resulting 3′-hydroxyl groups are transferred to target DNA (Craigie and Mizuuchi, 1987; Surette *et al.*, 1987; Mizuuchi *et al.*, 1992), producing a branched DNA structure with a potential replication fork at each Mu end.

A specific set of host proteins is required to replicate Mu DNA on this strand transfer intermediate, and MuA plays a key role in controlling access of host proteins to the two potential replication forks (Kruklitis and Nakai, 1994; Nakai and Kruklitis, 1995; Kruklitis *et al.*, 1996). Oligomeric MuA remains tightly bound to both Mu ends in a nucleoprotein complex known as the strand transfer complex (STC1) or type II transpososome (Surette *et al.*, 1987; Lavoie *et al.*, 1991). A group of host factors called Mu replication factors α (MRFα), which includes the molecular chaperone ClpX and at least one additional component ($MRF\alpha_2$) (Kruklitis *et al.*, 1996), removes MuA from STC1 to form a prereplisome, a nucleoprotein complex (STC3) that only allows initiation of Mu DNA synthesis by a specific set of host factors (Nakai and Kruklitis, 1995). These factors include replication proteins such as DnaB, DnaC and DNA pol III holoenzyme, which are known to be required for Mu DNA synthesis *in vivo*, and a group of host factors called MRFβ, previously used in the reconstituted system in partially purified form.

In this study, we identify the host factors in MRFβ as PriA, PriB and DnaT. We characterize the function of these proteins in promoting Mu replication on the Mu strand transfer intermediate.

Results

Mu replication by transposition in vivo is dependent on the priA gene function

We examined the ability of Mu to grow in *E.coli* strains with inactivating mutations in the *priA* gene. Two *E.coli* strains with *priA* null mutations (PriA⁻) supported Mu lysogenization but were unable to support lytic growth (Table I). The ability to support Mu lytic growth was restored by transformation with a plasmid expressing PriA (Table I).

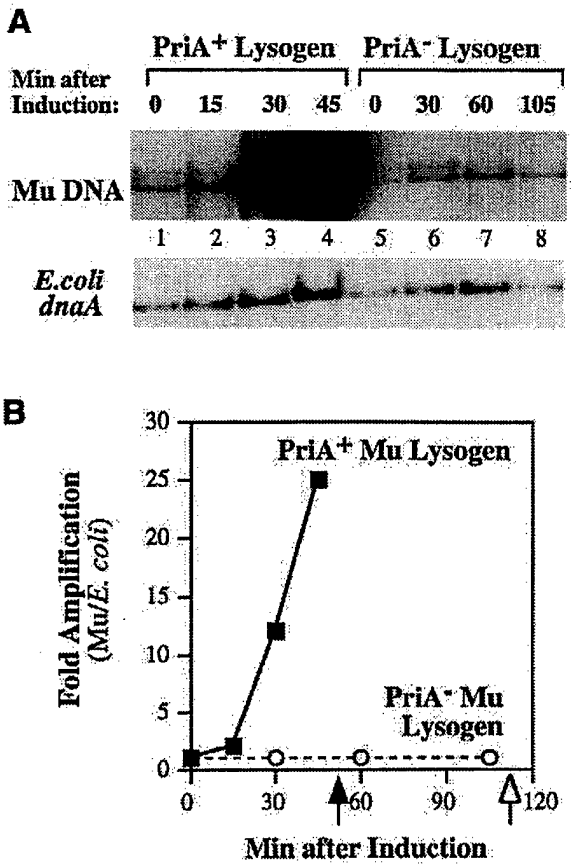

Fig. 1. Requirement for the *priA* function in bacteriophage Mu DNA replication *in vivo*. (**A**) Southern blot of DNA prepared from induced cultures of Mu lysogens AT3978 (PriA⁺) and AT3978 *priA1::kan* (PriA⁻) probed with Mu-specific and *E.coli dnaA*-specific sequences. (**B**) Quantitation of Mu DNA amplification relative to an *E. coli*-specific marker (*dnaA*). Solid and open arrows indicate the time at which lysis occurred for the PriA⁺ and PriA⁻ Mu lysogens, respectively.

To determine whether this block in lytic development specifically affected Mu replication by transposition, we examined amplification of Mu DNA in induced PriA⁺ and PriA⁻ Mu lysogens (*his*::Mucts62). Both lysogens eventually lysed after heat induction and, as expected, the PriA⁺ lysate was highly infectious [$>10^{10}$ plaque-forming units (p.f.u.) per ml] whereas the PriA⁻ lysate had no detectable titer ($<10^3$ p.f.u. per ml). Southern blot analysis of DNA isolated from the induced PriA⁺ Mu lysogen (Figure 1A, lanes 1–4) indicated that Mu DNA was amplified at least 25-fold relative to a host-specific marker (*dnaA*) before lysis (Figure 1B). No amplification was detected in the induced PriA⁻ lysogen (Figure 1A, lanes 5–8, and Figure 1B) even though reconstruction experiments indicated that as little as a 2-fold increase in Mu DNA could be detected using this Southern blot technique (data not shown). These results indicate that Mu was unable to undergo even one round of replication by transposition *in vivo* in the absence of PriA.

PriA and additional ϕX-type primosome constituents are required for Mu DNA replication in vitro

In the *in vitro* transposition system, STC1 is formed using a supercoiled plasmid bearing a mini-Mu element as donor

substrate and a second plasmid as target (Mizuuchi, 1983). Mu DNA in STC1 can be replicated to form a cointegrate using a reconstituted system composed of an eight-protein system [DnaB, DnaC, primase, DNA pol III holoenzyme, DNA pol I, DNA gyrase, single-strand binding protein (SSB) and DNA ligase] supplemented with MRFα (or ClpX and $MRF\alpha_2$) and MRFβ (Kruklitis and Nakai, 1994; Nakai and Kruklitis, 1995). MRFα and MRFβ can be supplied separately (each as fraction III) or together in a crude enzyme fraction (fraction II). We determined whether PriA was an essential component of this system.

The eight-protein system supplemented with fraction II from a $PriA^-$ *E.coli* strain did not support Mu DNA replication (Figure 2A). The addition of purified PriA restored only low levels of replication activity, while the addition of both PriA and DnaT restored activity to that obtained with fraction II from a wild-type strain, suggesting that Mu DNA replication was dependent on both PriA and DnaT and that our $PriA^-$ fraction II was also deficient in DnaT activity. Using a reconstituted assay for the replication of φX174 single-stranded DNA, we found that our $PriA^-$ fraction II was indeed partially deficient in DnaT activity relative to a fraction II from a $PriA^+$ strain (data not shown).

PriA was a necessary component of MRFβ which provides complementing activity in the reconstituted Mu replication system. While an MRFα fraction III prepared from a $PriA^-$ strain had complementing activity comparable with MRFα from a $PriA^+$ strain (data not shown), the MRFβ fraction III prepared from a $PriA^-$ strain showed only background levels of activity (Figure 2B). Unlike the $PriA^-$ fraction II, full activity was restored to MRFβ ($PriA^-$ fraction III) by the addition of purified PriA alone (Figure 2B). The specific activity of MRFβ is increased 10- to 15-fold during preparation of fraction III, and therefore the enrichment of low levels of DnaT in fraction II as well as removal of unwanted proteins most likely yielded a MRFβ($PriA^-$) fraction with sufficient DnaT activity to promote high levels of Mu DNA replication.

MRFβ could be replaced by purified PriA, PriB and DnaT (Figure 2C). Cointegrate production was absolutely dependent on PriA, DnaBC and MRFα as well as the φX components PriB and DnaT (Table II). The small amounts of cointegrate production apparent when either PriB or DnaT was omitted individually are most likely due to low levels of PriB and DnaT in the MRFα fraction, detected using the reconstituted φX174 replication assay (data not shown). The lack of any replication when both are omitted (Table II) strongly supports the conclusion that PriA is not acting independently of PriB and DnaT during Mu DNA replication but is assembling a multi-component primosome like the one characterized in φX174 replication. We could not determine the dependence of Mu replication on PriC because high levels of PriC activity were present in the MRFα fraction (data not shown). MRFα cannot be replaced with purified PriC and ClpX (Table II), indicating that at least one additional factor besides these two proteins is an essential MRFα component.

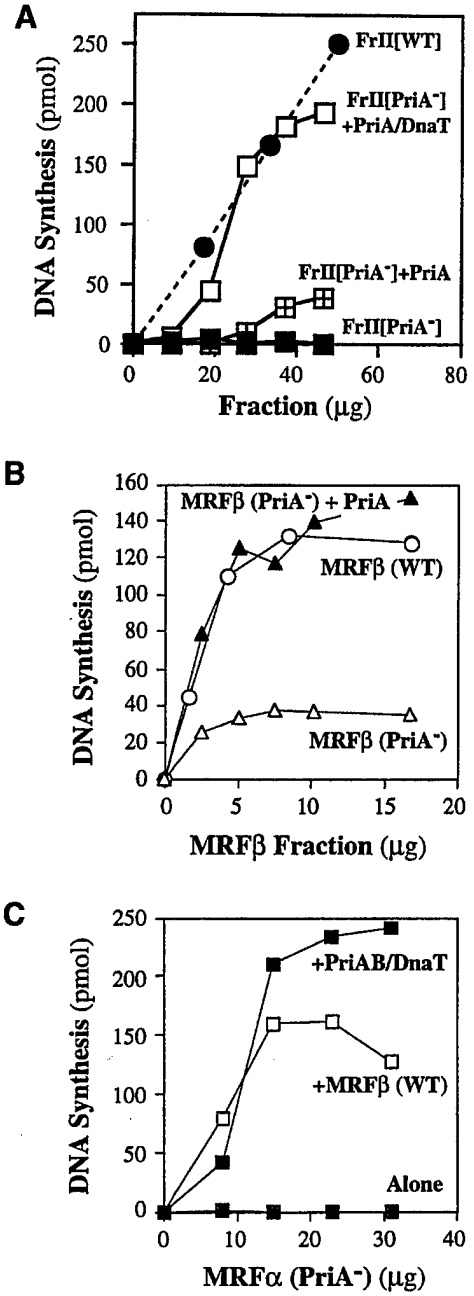

Fig. 2. Requirement for PriA and additional primosome proteins in the reconstituted Mu replication system. **(A)** Replication was catalyzed on STC1 (pXP10 target DNA) in the eight-protein system supplemented with the indicated proteins and with varying amounts of a crude enzyme fraction (fraction II) prepared from a $PriA^+$ (WT) or $PriA^-$ *E.coli* strain. **(B)** Replication was catalyzed on STC1 in the eight-protein system supplemented with MRFα, purified PriA, as indicated, and varying amounts of MRFβ prepared from $PriA^+$ (WT) or $PriA^-$ strains. **(C)** Replication was catalyzed on STC1 in the eight-protein system supplemented with MRFβ(WT) or purified PriA, PriB and DnaT, as indicated, and with varying amounts of MRFα($PriA^-$).

The φX-type primosome supports initiation of semi-discontinuous DNA synthesis with initial preference for the Mu left end

Replication of full-length (37 kb) Mu DNA in induced lysogens proceeds semi-discontinuously (Higgins *et al.*, 1983), with DNA synthesis *in vivo* initiating 80–90% of the time at the left end of full-length Mu (Wijffelman and van de Putte, 1977; Goosen, 1978; Pato and Waggoner, 1987). However, initiation of mini-Mu replication *in vivo* takes place at the left end only ~50% of the time (Harshey *et al.*, 1982; Résibois *et al.*, 1982a,b, 1984). We examined these properties in the reconstituted Mu replication system. To distinguish between leading and lagging strand syn-

Table II. Requirement for φX-type primosome components and MRFα in cointegrate formation

Component omitted[a]	pmol[b]	Co (%)[c]
None	185	100
MRFα	0	<1
MRFα (ClpX and PriC added)	0	<1
PriA	0	<1
DnaBC	0	<1
PriB and DnaT	0	<1
PriB	37	20
DnaT	14	8

[a]The complete reaction mixture included STC1 (pXP10 target DNA), the eight-protein system, MRFα(PriA⁻), PriA, PriB and DnaT, with omissions as indicated. Where indicated, ClpX (7.6 μg/ml) and PriC (0.8 U/ml) were also included.
[b]Total deoxynucleotide incorporation (pmol) was determined by counting one-tenth of each reaction mixture.
[c]The remaining products were linearized with *Nde*I and resolved on a 0.6% alkaline agarose gel. The amount of cointegrates was quantitated by phosphorimagery. The level of cointegrates formed in the complete reaction (no components omitted), in which >95% of the strand transfer products were converted to cointegrates, was set arbitrarily at 100.

thesis and between initiation at the Mu left and right ends, STC1 was replicated in a six-protein system (the eight-protein system lacking DNA pol I and ligase) supplemented with MRFα, PriA, PriB, PriC and DnaT. Products were digested with a restriction enzyme that cleaves within the donor vector near the Mu left end (Figure 3A). Leading strands corresponding to initiation at the left or right ends as well as Okazaki fragments from lagging strand synthesis could be distinguished by size on a denaturing agarose gel. To ensure examination of leading and lagging strand synthesis associated with cointegrate formation, linearized cointegrate products were first purified from a native agarose gel prior to separation by denaturing gel electrophoresis.

We confirmed the presence of short products (1–3 kb) consistent with lagging strand synthesis in the isolated cointegrate products (Figure 3B), with leading and lagging strand synthesis accounting for roughly equal amounts of nucleotide incorporation. The addition of DNA pol I and ligase shifted all products to the unit length of the cointegrate (Figure 3C), supporting the conclusion that the short products were indeed Okazaki fragments. Quantitation of the products of leading strand initiation from the left and right ends in these isolated cointegrates revealed only a small bias for initiation from the left end.

The relative frequency of leading strand synthesis initiating at the left and right ends of mini-Mu was determined *in vitro* in this experiment from all replication products that had accumulated at the completion of the reaction (30 min) and *in vivo* in previous work (Résibois *et al.*, 1984) from all products that had accumulated late in development. To determine whether earlier replication products *in vitro* reflect the left end bias seen with full-length Mu *in vivo*, we examined the kinetics of initiation at the left and right ends. Reactions were allowed to proceed for 5–30 min, and products were digested with restriction enzymes that cleave in the donor vector either very near the Mu left (*Bam*HI) or right end (*Nde*I) to distinguish leading strands corresponding to initiation at the left or right ends on a denaturing agarose gel (see Figure 3A). Full-length products corresponding to leading strand synthesis across the entire mini-Mu element were first evident at 10 min. Quantitation of cointegrate products digested with *Bam*HI or *Nde*I (Figure 4A) revealed that 90–100% of cointegrates formed at 10 min corresponded to initiation at the left end of Mu (Figure 4B), indicating that the initial rounds of replication do reflect a left end bias. Products of right end initiation accumulated more slowly, so that by 30 min they accounted for 25–45% of the products (Figure 4B). Thus, some feature of STC3 or the DNA template may permit the replisome to be assembled more readily at the left end. All of these results indicate that Mu DNA replication reconstituted with the φX174-type primosome reflects characteristics of Mu DNA replication observed *in vivo*.

φX-type primosome constituents promote engagement of DNA pol III holoenzyme on the recombined substrate

Mu DNA synthesis can initiate without MRFα, MRFβ, DnaB, DnaC and DNA pol III holoenzyme on the deproteinized strand transfer product (Kruklitis and Nakai, 1994; Nakai and Kruklitis, 1995), especially when DNA pol I (or the Klenow fragment) is present at high levels (Figure 5B, lane 1). We determined whether DNA pol III holoenzyme (prepared from a UvrD⁻ strain so that it is not contaminated with helicase II) can catalyze Mu DNA synthesis on the deproteinized strand transfer product when PriA, PriB and DnaT are absent. The deproteinized template was incubated for 15–60 min in the six-protein system (in the absence of DNA pol I and ligase), and products were cleaved within the donor vector (Figure 3A) so that extension from the two ends could be distinguished. Even after 30 min, no DNA synthesis was catalyzed on the deproteinized template in the six-protein system alone (Figure 5A, lane 1). When the six-protein system was supplemented with high levels of the DNA pol I Klenow fragment, extension of the leading strand primers at both ends proceeded slowly, consistent with the low processivity and distributive action of pol I. These primers were extended only 0.2–0.4 kb by 15 min (Figure 5A, lane 2), gradually being extended 1 kb or more by 60 min (Figure 5A, lane 5). Few or no products corresponding to complete replication of the mini-Mu element were formed even after 60 min. Moreover, the same level of DNA synthesis was catalyzed if DnaB and pol III holoenzyme were not present together with pol I (Figure 5B, lane 1). These results indicate that DnaB and DNA pol III holoenzyme are not engaged on the deproteinized template under these conditions.

However, when PriA, PriB, PriC and DnaT were added to the reaction mixture that included DNA pol I, full-length cointegrates were formed in 30 min (Figure 5B, lane 2). DNA ligase was included in these reactions so that full-length cointegrates could be easily distinguishable from the shorter, 30 min extension products of DNA pol I (Figure 5B, cf. Co and Ex). Quantitation of cointegrate production revealed that under these conditions at least 90% of the cointegrate products were dependent on not only PriA and DnaT but also on the DnaBC complex and pol III holoenzyme (Figure 5C). In separate experiments, we determined that cointegrate production was dependent on both DnaB and DnaC when they were

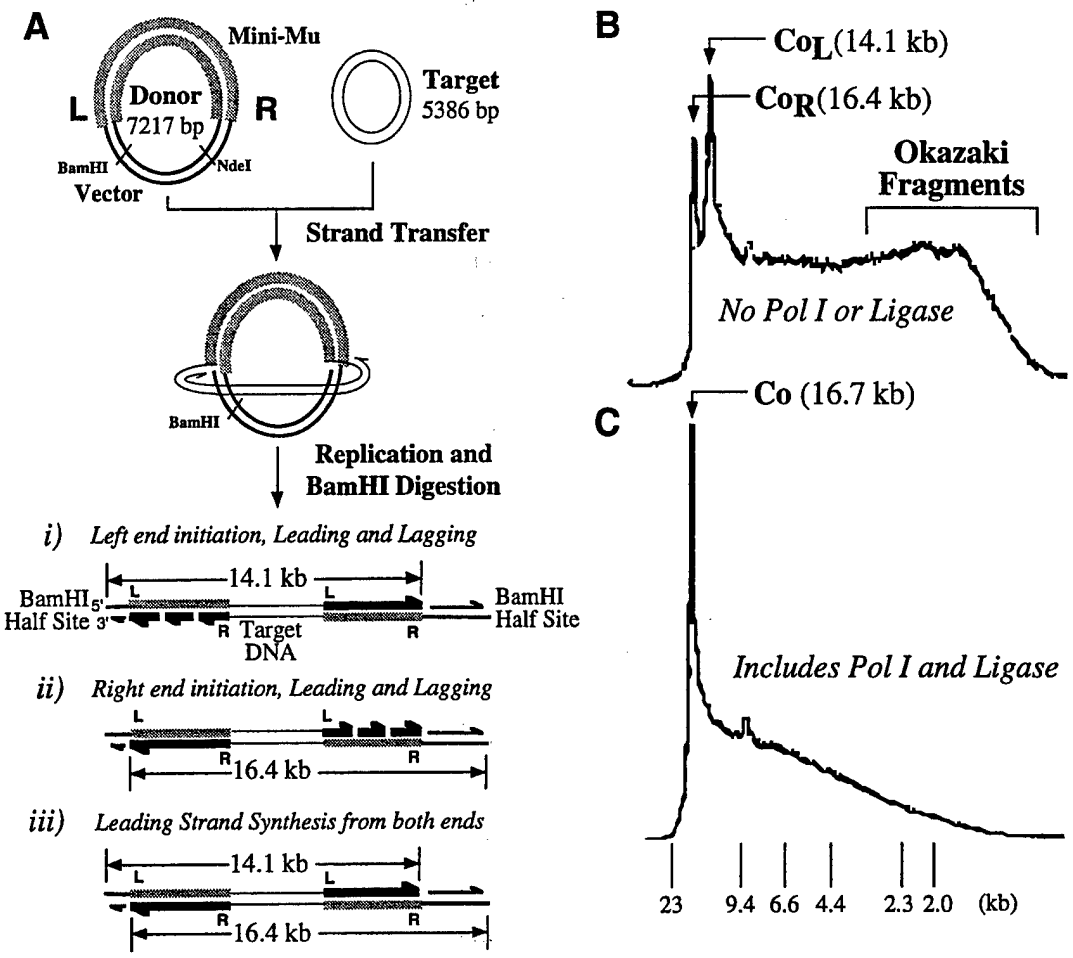

Fig. 3. Replication of STC proceeds by semi-discontinuous DNA synthesis. (**A**) *Bam*HI and *Nde*I cleave asymmetrically in the donor vector but not within the mini-Mu element or the transposition target. Cleavage of unligated replication products with one of these enzymes (e.g. *Bam*HI) results in a unique series of labeled DNA fragments whose lengths depend on the mode of replication: initiation of leading and lagging strand synthesis from the left (*i*) or right ends (*ii*) or initiation of leading strand synthesis from the primers at both ends (*iii*). (B) and (C) Replication on STC1 (ϕX174 RFI target DNA) was conducted in the six-protein system (lacking DNA pol I and ligase) (**B**) or the eight-protein system (**C**) supplemented with MRFα(PriA$^-$), PriA, PriB and DnaT. Full-length cointegrate products linearized with *Bam*HI were purified by native gel electrophoresis and then resolved on a 0.6% alkaline agarose gel, which was dried for phosphorimagery. Linear scans of the radiolabeled products in each lane are shown. Peaks corresponding to unit length cointegrate (Co), leading strand products resulting from initiation at the Mu right (Co_R) and left (Co_L) ends and products of lagging strands synthesis were identified based on their migration relative to molecular weight standards.

added individually (data not shown). Therefore, the PriA-dependent replication pathway engages DnaB helicase and pol III holoenzyme to replicate Mu DNA rapidly on the strand transfer product.

Extension of the leading strand primer by DNA pol I is not essential for PriA-dependent DNA synthesis on the Mu strand transfer intermediate

In pBR322 replication, an RNA polymerase transcript that primes DNA synthesis at the origin must be extended by DNA pol I to form a D-loop and expose a PAS on the displaced single strand to maximize PriA-promoted assembly of the pre-primosome (Minden and Marians, 1985). On the Mu strand transfer intermediate, there is no single-stranded region on the lagging strand side of each fork potentially to serve as a binding site for the pre-primosome (see Figure 7A). Although DNA pol I can extend the leading strand at each Mu end of the deproteinized template to expose single-stranded DNA, it was not essential for PriA-dependent cointegrate formation (Figure 5C). Its presence did increase the level of nucleotide incorporation and cointegrate formation by ~2-fold, suggesting the possibility that the efficiency of preprimosome assembly can be maximized by limited extension of the leading strand primers.

When DNA synthesis was catalyzed on STC1, the leading strand primers were not extended at all unless all required replication proteins including PriA, DnaT and MRFα were present (Figure 6, lane 1). When PriA or DnaBC was omitted, no cointegrates were formed, and the leading strand primers could not be extended by high levels of DNA pol I (Figure 6, lanes 2 and 3) as they were on the deproteinized template (lane 4). Whereas 400–500 nucleotides were incorporated per deproteinized template in 30 min, the amount of nucleotide incorporation during this time on the STC without PriA or DnaBC was below detectable levels, which correspond to <10 nucleotides being incorporated per template. This level of nucleotide incorporation by itself is unlikely to produce a duplex opening sufficient to promote primosome assembly. When the DNA duplex at a ColE1-type plasmid origin is opened by an R-loop, a single-stranded region with a

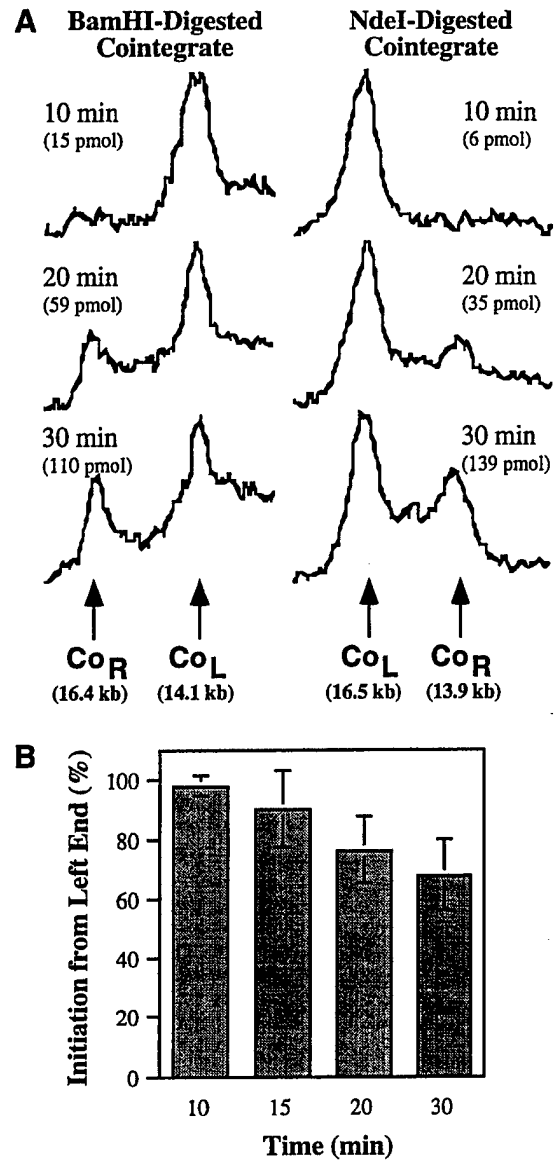

Fig. 4. Replication on STC initiates preferentially from the left end of Mu. (**A**) Replication on STC1 (ϕX174 RFI target DNA) was allowed to proceed for 5–30 min in the six-protein system (lacking DNA pol I and ligase) supplemented with MRFα(PriA$^-$), PriA, PriB and DnaT. Cointegrate products were linearized with *Bam*HI or *Nde*I and resolved on a 0.6% alkaline agarose gel, which was dried for phosphorimagery. Linear scans of the radiolabeled leading strand products from the 10, 20 and 30 min reactions are shown. Peaks corresponding to leading strand products resulting from initiation at the Mu right (Co_R) and left (Co_L) ends were identified based on their migration relative to molecular weight standards. Total deoxynucleotide incorporation (pmol) in each reaction is indicated; scans have been normalized for total cointegrate formation. (**B**) The percentage of total leading strand synthesis initiating at the Mu left end was quantitated by phosphorimagery. Results are the average of three independent trials, including one in which products were digested with *Nde*I and two in which products were digested with *Bam*HI; standard deviation of the mean is indicated by error bars.

minimum of 40 bases must be exposed to activate DNA synthesis in the absence of DNA pol I (Masukata *et al.*, 1987). Together with previous findings that the polymerase activity of DNA pol I is not required to initiate DNA synthesis on STC (Kruklitis and Nakai, 1994), our results indicate that the leading strand primer is not extended before assembly of the preprimosome on the STC and initiation of PriA-dependent Mu DNA synthesis.

Discussion

Mechanism for replisome assembly during Mu transposition

Bacteriophage Mu DNA synthesis by transposition requires a specific set of replication proteins (including DnaB helicase, DnaC protein, primase and DNA pol III holoenzyme) known to be required for initiation at *oriC* (Kaguni and Kornberg, 1984). Because initiation of Mu DNA synthesis does not require the DnaA protein (McBeth and Taylor, 1982; Kruklitis and Nakai, 1994), a major question has been how these proteins are assembled into a replisome once the recombination portion of the reaction has been carried out by the Mu transposition apparatus. The function of PriA, PriB and DnaT in Mu DNA synthesis characterized in this work and the previously characterized properties of the ϕX-type primosome indicate how these specific replication proteins are engaged for replicative transposition.

The transition from transpososome to replisome illustrates how the complex series of reactions needed for Mu replication are promoted sequentially through remodeling of nucleoprotein complexes at the Mu ends. STC1 is converted to STC2 by the action of the chaperone ClpX coupled to ATP hydrolysis (Kruklitis *et al.*, 1996), altering MuA quaternary structure (Levchenko *et al.*, 1995) and activating the transpososome's potential to promote transition to DNA replication. In a second ATP-dependent reaction, MRFα_2 displaces MuA in STC2 to form the prereplisome STC3, which only permits initiation of DNA synthesis by the specific group of replication proteins including MRFβ (Nakai and Kruklitis, 1995; Kruklitis *et al.*, 1996).

Our identification of MRFβ as PriA, PriB and DnaT makes evident the probable sequence of events that lead to replisome assembly for Mu DNA synthesis. In ϕX174 complementary strand synthesis, PriA binds to the PAS to begin the assembly process (Wickner and Hurwitz, 1975; Shlomai and Kornberg, 1980; Ng and Marians, 1996a). PriB and DnaT join the PriA–PAS complex, and then DnaB is delivered from the DnaB–DnaC complex to form the preprimosome (Ng and Marians, 1996a). Thus, PriA is the likely component that first assembles on STC3 or the deproteinized strand transfer intermediate, initiating the assembly sequence that leads to preprimosome assembly (Figure 7A–C). Our finding that PriA-dependent DNA synthesis on the deproteinized strand transfer intermediate could be catalyzed at lower levels without PriC or PriB was not surprising. PriC can be dispensable for primosome assembly and ϕX174 DNA synthesis (Ng and Marians, 1996a). Although PriB promotes interaction between PriA and DnaT, the PriA–DnaT complex on DNA can be formed at high DnaT concentrations in the absence of PriB (Liu *et al.*, 1996). DnaB in the preprimosome can recruit the two other specific enzymes needed to propagate the Mu replication fork. DnaB, through its specific interaction with the τ subunit of DNA pol III holoenzyme, can promote stable binding of this dimeric polymerase on the leading strand of the fork (Yuzhakov *et al.*, 1996), thus recruiting simultaneously

the polymerase for leading and lagging strand synthesis (Figure 7D). DnaB helicase can also attract primase (Tougo *et al.*, 1994) to initiate lagging strand synthesis (Figure 7E).

Our results indicate that PriA plays a crucial function in assembling a replisome on a recombination intermediate. A question raised by these studies is what constitutes a PAS on the Mu strand transfer intermediate. The pre-replisome STC3 allows only PriA-dependent Mu DNA synthesis to proceed, and the factors that play this gate-keeper role could stabilize a DNA structure that serves as a PAS. Even though these factors are not essential to engage PriA on this template, STC1 is replicated approximately twice as fast as the deproteinized template under identical reaction conditions (data not shown). Another important consideration is that the leading strand primers of STC3 cannot be extended to open the duplex prior to engagement of PriA. Thus, duplex opening at the Mu ends by DNA pol I cannot be the mechanism for creating a PriA-binding site. Instead, some feature of the DNA structure of a strand transfer intermediate may be important for initial PriA binding, which leads to duplex opening and primosome assembly. Recent evidence that PriA can bind to D-loops and DNA structures that resemble the branched structure of the strand transfer intermediate at each Mu end (McGlynn *et al.*, 1997) supports this hypothesis.

The left end bias observed in the initiation of Mu DNA replication *in vivo* and *in vitro* may reflect asymmetry of the STC in providing PriA-binding sites at the left and right ends. Such an asymmetry could be due to the presence of a strong PAS at or near the Mu left end. However, what would constitute a PAS on a branched recombination intermediate and how it may be structurally related to the PAS on the ϕX174 template are not yet clear.

Relevance to understanding the host system for coupling recombination with DNA replication

Kogoma (Asai and Kogoma, 1994; Kogoma, 1996) has hypothesized that DNA replication plays an important role in recombinant formation by homologous recombination and that the ϕX-type primosome plays a key role in assembling replisomes on recombination intermediates. Our results support this hypothesis and suggest that the Mu transposition apparatus ensures efficient replication of the Mu genome by specifically recruiting the host apparatus that links recombination with replication.

For replication linked to both Mu transposition and homologous recombination, replisome assembly would be coordinated with molecular events and signals different from those which control replisome assembly at *oriC*. While DnaA coordinates initiation with the cell cycle, our results indicate that PriA can respond to molecular signals on a recombination intermediate to initiate replisome assembly, a critical function in linking recombination with DNA synthesis.

In the Mu system, access of the potential replication forks to host proteins is carefully restricted. PriA can promote initiation only upon conversion of STC1 to STC3

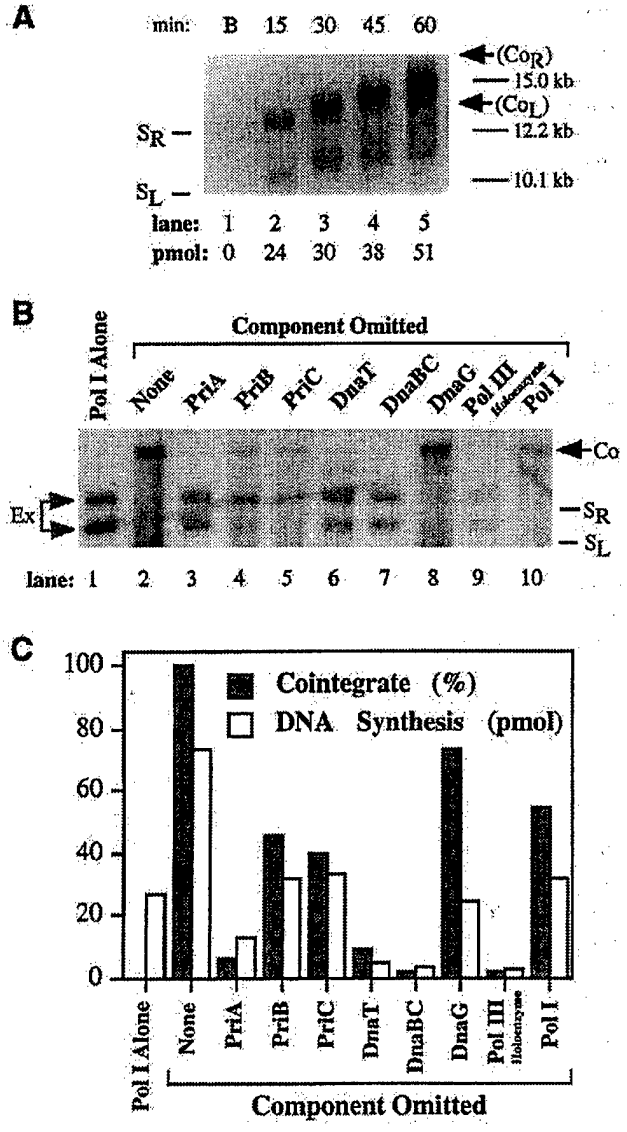

Fig. 5. ϕX-type primosome constituents promote engagement of DNA pol III holoenzyme on the deproteinized strand transfer product. (**A**) Replication was conducted on the deproteinized strand transfer product (ϕX174 RFI target DNA) in the six-protein system supplemented with the DNA pol I Klenow fragment (100 U/ml) for 15–60 min (lane 1: six-protein system alone, 30 min). Products were digested with *Bam*HI and resolved on a 0.6% alkaline agarose gel. The length of the replication products increases with time as the leading strand primers are slowly extended by Klenow. Total deoxynucleotide incorporation (pmol) in each reaction is indicated. For reference, the positions of unextended leading strand primers from the strand transfer intermediate (S) and of fully extended leading strands from the co-integrate (Co) resulting from initiation at the Mu left (Co_L, S_L) and right (Co_R, S_R) ends are indicated; replication products in this reaction did not reach full length. (**B**) Replication was conducted on the deproteinized strand transfer product (f1 RFI target DNA) in the eight-protein system supplemented with PriA, PriB, PriC, DnaT and additional DNA pol I (2 U/ml). Proteins were omitted as indicated (lane 1: replication by 2 U/ml DNA pol I in the absence of DnaBC, PriABC, DnaT and DNA pol III). Products were digested with *Eco*RI and resolved on a 0.6% alkaline agarose gel. Positions of the full-length cointegrate (Co), unreplicated strand transfer intermediates (S_L and S_R) and leading strand primers extended by DNA pol I (Ex) are shown. (**C**) Total deoxynucleotide incorporation (pmol) was determined by counting one-tenth of each reaction mixture (white bars). The remaining products were resolved on a 0.6% alkaline agarose gel. The amount of cointegrates was quantitated by phosphorimagery (shaded bars). The level of cointegrates formed in the complete reaction (no components omitted), in which ~60% of the strand transfer products were converted to cointegrates, was set arbitrarily at 100. Results are the average of two independent experiments.

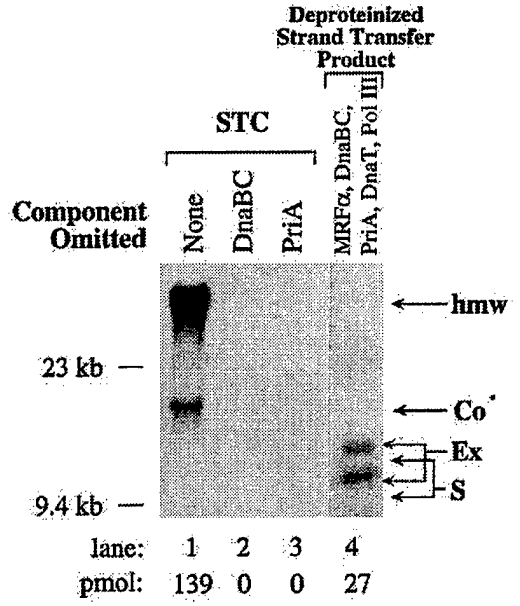

Fig. 6. Leading strand primers at the ends of Mu in STC are not extended in the absence of PriA. Replication was conducted on STC1 (f1 RFI target) in the eight-protein system supplemented with PriA, PriB, PriC, DnaT, MRFα(PriA⁻) and additional DNA pol I (2 U/ml). Proteins were omitted as indicated (lane 4: replication of deproteinized strand transfer product by 2 U/ml DNA pol I in the absence of MRFα, DnaBC, PriABC, DnaT and DNA pol III, 30 min). Products were digested with *Eco*RI and resolved on a 0.6% alkaline agarose gel. Positions of the full-length cointegrate (Co), unreplicated strand transfer intermediates (S) and leading strand primers extended by DNA pol I (Ex) are shown.

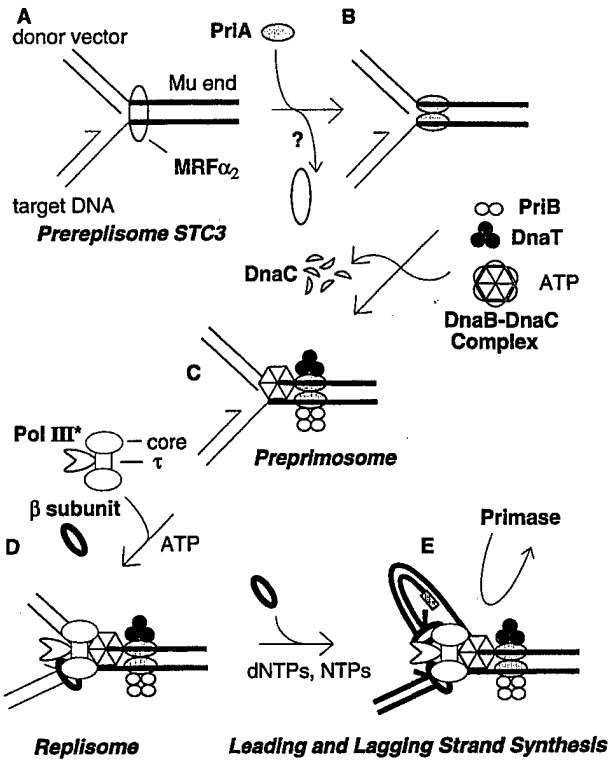

Fig. 7. Model for replisome assembly at the site of Mu strand exchange. Action of ClpX and MRFα$_2$ converts the transpososome STC1 to the prereplisome STC3 (**A**). In this complex, MuA has been removed from the Mu ends (one end is shown), forming a new nucleoprotein complex that does not permit the leading strand to be extended by DNA pol I (Kruklitis *et al.*, 1996). Even though there is no single-stranded segment on the lagging strand side of the fork, PriA binds to the pre-replisome (**B**), perhaps binding to a branched structure or a duplex opening stabilized by the prereplisome. Upon assembly of the preprimosome (**C**), DnaB promotes stable binding of DNA pol III* (holoenzyme minus the β subunit) to the leading strand primer (**D**) through interactions with the τ subunit (Yuzhakov *et al.*, 1996). The composition of the preprimosome is preserved (Ng and Marians, 1996b) as its helicase activity unwinds duplex DNA for leading strand synthesis. Its transient interaction with primase (Ng and Marians, 1996a) forms the primosome, catalyzing primer synthesis and initiating DNA synthesis by the lagging strand polymerase of dimeric pol III*.

by action of ClpX and MRFα$_2$ (Nakai and Kruklitis, 1995; Kruklitis *et al.*, 1996). This strategy may also be employed in homologous recombination. MRFα$_2$, which is involved in converting STC2 to STC3, may similarly be involved in controlling access of host proteins to D-loops, promoting PriA-dependent DNA replication. Not all homologous recombination requires PriA, suggesting that intermediates formed by strand exchange can be resolved with or without DNA replication (Kogoma *et al.*, 1996). Cellular factors may control the decision whether or not to assemble a replisome.

Thus, an intriguing question is how the engagement of PriA on a recombination intermediate would be regulated to control initiation. PAS sequences are underrepresented on the *E.coli* chromosome (Stuitje *et al.*, 1984), and at *oriM1*, the origin for iSDR in the *oriC* region, no PAS can be found by functional assays within the vicinity of ~2.5 kb (Stuitje *et al.*, 1984; Asai and Kogoma, 1994). It is therefore likely that signals other than the ϕX174-type PAS, DNA structures created during recombination and possibly stabilized by MRFα$_2$ or related cellular factors, play a key role in engagement of PriA. Through control of PriA action, the fate of a recombination intermediate can be determined, a process vital for the maintenance of the bacterial chromosome.

Materials and methods

Bacterial and bacteriophage strains and proteins

Escherichia coli strains EL500 (*priA1::kan, recD::mini-tet*), EL501 (pEL042 expressing wild-type *priA, recD::mini-tet*) and EL502 (pEL042, *priA1::kan, recD::mini-tet*) have been described (Lee and Kornberg, 1991). AT3327 *priA1::kan* and AT3978 *priA1::kan* were constructed by introducing *priA1::kan* into AT3327 (*mal*) and AT3978 (Hfr PK191 *his::*Mucts62pAp1), respectively, by P1 transduction. Mucts62pAp1, which carries a determinant for ampicillin resistance (Leach and Symonds, 1979), was grown by heat induction of AT3978.

DNA pol III* was purified from MGC1020 (W3110 *malE::Tn10, lexA3, uvrD::kan*) obtained from Dr Charles McHenry (University of Colorado Health Sciences Center) as previously described (Maki *et al.*, 1988). PriA, PriB, PriC and DnaT were purified from overproducing strains to >95% homogeneity as described (Marians, 1995). Purified preparations of these four proteins used for initial studies were kindly provided by Dr Arthur Kornberg (Stanford University School of Medicine). DNA pol I and the DNA pol I large (Klenow) fragment were purchased from New England BioLabs. All other proteins were purified as previously described (Kruklitis and Nakai, 1994; Nakai and Kruklitis, 1995; Kruklitis *et al.*, 1996).

Mu growth in vivo

To compare the plating efficiency of PriA$^+$ and PriA$^-$ bacterial strains, Mu *cts62*pAp1 was titered on various indicator strains which were seeded in soft agar on L broth plates. The number of p.f.u. per ml was determined after incubation of the plates overnight at 37°C. Relative plating efficiencies, with the titer on EL501 and AT3327 arbitrarily set to 1, were calculated from the averages of three independent trials; standard errors of the mean were <50%. To measure lysogenization frequency, indicator strains were infected with serial dilutions of Mu*cts62*-

pAp1, and cells were plated on L broth plates supplemented with ampicillin (50 μg/ml), incubated overnight at 30°C and scored for ampicillin-resistant colonies (Mu lysogens). Lysogenization frequency was calculated as the number of lysogens per p.f.u. Values shown are the average of three independent trials; the standard errors of the mean were <50%. Plating assays indicated that PriA⁻ strains had a 5- to 10-fold reduced viability relative to wild-type strains as observed elsewhere (Kogoma *et al.*, 1996); however, lysogenization frequencies were not corrected for this.

Mu DNA replication in vivo

To measure the level of Mu DNA replication by transposition *in vivo*, lysogens AT3978 (*his*::Mu *cts62*pAp1) and AT3978 *priA1::kan* were grown at 30°C to early log phase (OD_{600} = 0.4) and then incubated at 42°C until lysis occurred. Cultures were sampled at various times after the shift to 42°C. Cell growth in the samples was stopped by the addition of 10 mM sodium azide. RNase-treated genomic DNA from these samples (2.0 μg each) was digested to completion with *Eco*RI, separated on a 0.6% agarose gel (TAE electrophoresis buffer: 40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 8.1), transferred to a nylon membrane (ICN Biotrans™) by alkaline capillary transfer (Selden, 1992) and probed with ^{32}P-labeled Mu DNA (500 000 c.p.m. per lane) from phage grown in *Proteus mirabilis*. The blot was stripped for 2 h at 75°C (1 mM Tris, 1 mM EDTA, 0.002% each of bovine serum albumin, polyvinylpyrrolidone and Ficoll 400, pH 8.0) and reprobed with ^{32}P-labeled pKA211 (from Dr Tsutomo Katayama, Georgetown University), which contains the *E.coli dnaA* gene located near *oriC* (Kornberg and Baker, 1992). Both probes were labeled to high specific activity (>2×10^8 c.p.m./μg) by nick translation (Sambrook *et al.*, 1989). The relative amplification of Mu over the *dnaA* gene was measured using the Molecular Dynamics Storm 840 phosphorimager system.

Mu DNA replication in vitro

Mu DNA synthesis was conducted on STC1 or the deproteinized strand transfer product (equivalent of 0.25 μg donor substrate), which was prepared as previously described (Nakai and Kruklitis, 1995) using pGG215 donor substrate (Surette *et al.*, 1987) and three different targets: pXP10 plasmid (Nakai and Kruklitis, 1995), ϕX174 RFI DNA and f1 RFI DNA (f1 contains no PAS; Zipursky and Marians, 1980). Where indicated, reaction mixtures (50 μl) contained crude *E.coli* enzyme fractions (fraction II) or fraction III of MRFα and MRFβ (240 U/ml of each unless otherwise indicated) prepared from *E.coli* strains WM433 (PriA⁺) or AT3327 *priA1::kan* (PriA⁻) as previously described (Nakai and Kruklitis, 1995). Purified proteins used in the reconstituted Mu replication system included PriA, PriB, PriC, DnaT (0.8 U/ml each; see Marians, 1995, for unit definition) and the eight-protein system composed of DNA gyrase (6.7 μg/ml), DnaB–DnaC complex (1.3 μg/ml), DnaG (0.84 μg/ml), DNA pol III holoenzyme (1.16 μg/ml), SSB protein (0.9 μg/ml), DNA pol I (0.2 U/ml) and DNA ligase (4 U/ml), or the six-protein system, which consisted of the same proteins except pol I and ligase. Reaction conditions and determination of total deoxynucleotide incorporation were as previously described (Nakai and Kruklitis, 1995). Reaction mixtures were incubated at 37°C for 30 min unless otherwise indicated. For quantitation of cointegrate production by phosphorimagery, reaction products were deproteinized and digested with *Bam*HI or *Nde*I (ϕX174 RFI target), *Nde*I (pXP10 target) or *Eco*RI (f1 RFI target). All of these enzymes cut once in the donor vector to linearize the cointegrate product. Products were then separated on a 0.6% alkaline agarose gel (Sambrook *et al.*, 1989).

For examination of leading and lagging strand synthesis and quantitation of initiation at the Mu left and right ends, the six-protein system was used and 1 mg/ml nicotinamide adenine mononucleotide (an *E.coli* ligase inhibitor) replaced nicotinamide adenine dinucleotide. This prevented the nick at the end of each leading strand from being sealed and prevented ligation of Okazaki fragments into a continuous strand. Products were deproteinized, digested with enzymes that cut once in the donor vector near either the left (*Bam*HI) or right (*Nde*I) Mu end and resolved on a 0.6% alkaline agarose gel. Where indicated, linearized cointegrates were first isolated on a native 0.6% agarose gel (TAE electrophoresis buffer) and purified using the GLASSMAX® DNA Isolation Matrix System (Gibco-BRL Life Technologies) before resolving on the alkaline gel.

Alkaline agarose gels were stained with SYBR® Green I nucleic acid stain for imaging and dried down for phosphorimagery on the Molecular Dynamics Storm 840 system. All quantitative data were analyzed using ImageQuant software. All images in the figures are from autoradiographs.

Acknowledgements

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Duplex Opening by Primosome Protein PriA for Replisome Assembly on a Recombination Intermediate

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PriA and other primosome assembly proteins of *Escherichia coli* recruit the major replicative helicase DnaB for replisome assembly during bacteriophage Mu transposition and replication. MuA transposase catalyzes the transfer of Mu ends to target DNA, forming a potential replication fork that provides the assembly site for the replisome. However, this fork lacks the single-stranded DNA needed to load DnaB. Although no pre-existing primosome assembly sites that bind PriA were found within the Mu end sequences, PriA was able to bind to the forked DNA structure created by MuA. The helicase activity of PriA could then open the duplex to create the DnaB binding site. In a tightly coupled reaction on synthetic forked substrates, PriA promoted both the unwinding of the lagging strand arm and preprimosome assembly to load DnaB onto the lagging strand template. PriA apparently translocated 3′ to 5′ along the lagging strand template until sufficient single-stranded DNA was exposed for binding of DnaB, which then translocated 5′ to 3′ in the opposite direction. Mutant PriA lacking helicase activity was unable to promote this process, and loss of PriA helicase impaired Mu DNA replication *in vivo* and *in vitro*. This suggests that the opening of the duplex by PriA helicase is a critical step in the initiation of Mu DNA replication. Concerted helicase and primosome assembly functions would allow PriA to act as initiator on recombination intermediates and stalled replication forks. As part of the replisome, PriA may act as a mobile initiator that minimizes interruptions in chromosomal replication.

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Keywords: in vitro DNA replication; phage Mu; PriA helicase; primosome

Introduction

Bacteriophage Mu DNA replication by transposition is a process intimately linked to non-homologous strand exchange catalyzed by MuA transposase. Monomeric MuA (Kuo *et al.*, 1991) binds to specific sites at the Mu ends (Craigie *et al.*, 1984), assembling into an active oligomeric transpososome bound to both Mu ends (Figure 1(a); Lavoie *et al.*, 1991; Mizuuchi *et al.*, 1992) aided by the host HU protein (Craigie *et al.*, 1985; Lavoie & Chaconas, 1993, 1994). Tetrameric MuA in the transpososome (Lavoie & Chaconas, 1990; Surette *et al.*, 1987) produces nicks at the Mu ends (Figure 1(b)) that are transferred to target DNA (Craigie & Mizuuchi, 1985, 1987) bound with a second transposition protein MuB (Adzuma & Mizuuchi, 1988). The resulting product, strand transfer complex 1 (STC1; Figure 1(c)), includes a potential replication fork (the Mu fork) that can act as the initiation site for Mu DNA replication at each Mu end, with the 3′-OH ends of target DNA providing the primers for leading strand synthesis. However, access to these forks is restricted by oligomeric MuA which remains tightly bound to the Mu ends (Kruklitis & Nakai, 1994).

In preparation for replisome assembly, MuA promotes the formation of a prereplisome at the Mu fork (Nakai & Kruklitis, 1995). First, the molecular chaperone ClpX alters quaternary interactions of oligomeric MuA (Levchenko *et al.*, 1995) in STC1, forming an altered transpososome STC2 (Kruklitis *et al.*, 1996). Additional host factors (Mu replication factor α_2 or MRFα_2) then displace MuA from STC2 to form a new nucleo-

Abbreviations used: STC, strand transfer complex; MRF, Mu replication factor; pol, polymerase; PAS, primosome assembly site; RF, replicative form; WT, wild-type.

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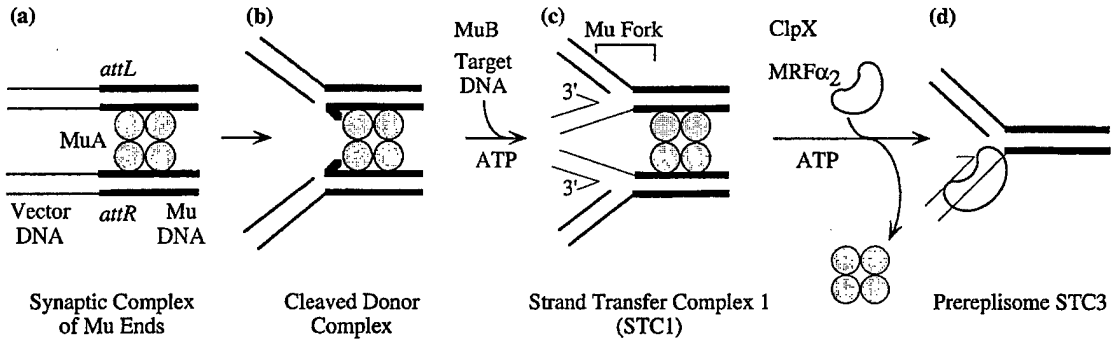


Figure 1. Formation of the transpososome and prereplisome during Mu transposition. (a) The transposase MuA binds to sites on the left (*attL*) and right (*attR*) ends of Mu (thick lines) forming a tetramer that brings the ends together in a synaptic complex. (b) The transpososome consisting of tetrameric MuA introduces a nick at each Mu end. (c) The resulting 3′-OH ends (half arrows) are transferred to target DNA (very thin lines) bound with MuB to form STC1, a complex which includes a potential replication fork (the Mu fork) at each Mu end. (d) Molecular chaperone ClpX changes the quaternary interactions within the MuA tetramer to form STC2 (not shown) and factors within the partially purified host fraction $MRF\alpha_2$ displace MuA from STC2, disrupting the synaptic complex of Mu ends and forming the prereplisome STC3 (only one Mu end is shown).

protein complex STC3 (Figure 1(d)), a prereplisome that permits Mu DNA synthesis only by specific primosome components and the DNA polymerase (pol) III holoenzyme (Jones & Nakai, 1997; Kruklitis *et al.*, 1996; Nakai & Kruklitis, 1995).

The disassembly of the transpososome leads to the formation of a replisome that catalyzes semi-discontinuous DNA synthesis from one Mu end to the other to form the cointegrate replication product. Central to this process is the assembly of a preprimosome at the Mu fork using the proteins PriA, PriB, PriC, DnaT, DnaB, and DnaC (Jones & Nakai, 1997). Together with primase these proteins comprise the ϕX174-type primosome, originally characterized as an apparatus that primes DNA synthesis on the single-stranded ϕX174 template (Wickner & Hurwitz, 1974). After the major replicative helicase DnaB is loaded from the DnaB-DnaC complex onto the lagging strand template, DnaB can serve as the organizing center of the replisome, stably binding the dimeric DNA pol III holoenzyme to the leading strand primer (Yuzhakov *et al.*, 1996). DnaB translocates 5′ to 3′ along the lagging strand template to unwind the helix (LeBowitz & McMacken, 1986) and attracts primase for lagging strand synthesis (Tougo *et al.*, 1994).

PriA, PriB, PriC, and DnaT's function in promoting the binding of DnaB at the fork distinguishes the process from DnaB assembly at the bacterial origin *oriC* where the initiator protein DnaA plays this role (Funnell *et al.*, 1987). PriA and the other primosome components have been found to play an important function in the initiation of DnaA-independent DNA synthesis such as replication of pBR322 (Minden & Marians, 1985). PriA-deficient strains assimilate genetic markers poorly by homologous recombination and are defective in double-strand break repair as well as inducible and constitutive stable DNA replication (Kogoma *et al.*, 1996; Masai *et al.*, 1994). Thus, PriA may couple homologous recombination to DNA replication by promoting replisome assembly at D-loop structures created by homologous strand exchange (Asai & Kogoma, 1994; Kogoma, 1996), a function that would also allow PriA to promote restart of DNA replication when replication forks stall.

PriA is the primosome component that initially binds to the DNA template. ϕX174 DNA includes a single primosome assembly site (PAS), a sequence to which PriA binds (Arai & Kornberg, 1981; Shlomai & Kornberg, 1980a), and there are two PASs near the origin of pBR322 (Zipursky & Marians, 1980). PriA can also bind to branched DNA structures that resemble D-loops (McGlynn *et al.*, 1997). We have previously demonstrated that PriA promotes primosome-dependent Mu DNA replication on both the prereplisome STC3 and the strand transfer product deproteinized by extraction with phenol (Jones & Nakai, 1997). However, the potential replication fork created by strand transfer does not include single-stranded DNA on the lagging strand arm. This poses a problem for the loading of DnaB, which occupies 20 nt of single-stranded DNA on the lagging strand template (Bujalowski & Jezewska, 1995). The 3′ to 5′ helicase activity of PriA (Lasken & Kornberg, 1988; Lee & Marians, 1987) could potentially create the necessary duplex opening, analogous to DnaA opening the duplex at *oriC* to create a binding site for DnaB (Bramhill & Kornberg, 1988).

Here, we investigate the function of PriA helicase in Mu DNA replication, establishing a new role for PriA's helicase activity in catalyzing a critical step in initiation. While the PriA helicase activity is not needed for primosome assembly on single-stranded templates (Zavitz & Marians, 1992), we demonstrate that it can open the duplex for entry of DnaB when sufficient single-stranded DNA is not available.

Results

PriA helicase can catalyze a critical step in the initiation of Mu DNA replication

PriA is essential *in vivo* for Mu DNA replication by transposition. No phage growth and no measurable amplification of Mu DNA can be detected in a *priA1::kan* host (Jones & Nakai, 1997). We determined that the mutant PriA K230R protein, which is defective in 3′ to 5′ helicase activity (Zavitz & Marians, 1992), was partially deficient in its ability to support Mu DNA replication. Mu plating efficiency was reduced by 50% on a PriA K230R host (data not shown), and PriA K230R promoted relatively slow rates of Mu DNA replication *in vivo*. AT3853 *priA1::kan*, a thermoinducible Mucts62 lysogen, was transformed with a plasmid expressing either PriA (pEL042; Lee & Kornberg, 1991) or PriA K230R (pEL042 K230R), and the lysogens were induced at mid-exponential phase (1.5×10^8 cells/ml). Phage development was monitored by quantitating amplification of Mu DNA relative to a host marker by Southern blot analysis and by scoring phage production. In the PriA K230R strain, Mu DNA was replicated at a reduced rate and was amplified to less than 30% the level attained in the PriA$^+$ strain (Figure 2(a)). Under these growth conditions, release of phage particles from the PriA K230R strain was delayed 20-30 minutes with a burst size approximately 50% that of the PriA$^+$ lysogen (Figure 2(b)). When cultures were diluted 20-fold at the start of induction, the difference in phage yield between the PriA$^+$ and PriA K230R strains was decreased (Figure 2(c)). These results indicate that PriA helicase is required for optimal rates of Mu replication *in vivo*, especially for phage growth at higher cell densities.

The strand transfer complex STC1 can be converted to a cointegrate *in vitro* in a system containing the φX-type primosome components, DNA pol III holoenzyme, SSB, DNA gyrase, ClpX and the host fraction MRFα (Jones & Nakai, 1997). If PriA K230R replaced PriA in this system, cointegrate production would be reduced as much as 50-fold (Figure 3(a), cf. lanes 5 and 9). Other proteins present in a crude cell extract, however, could complement the helicase defect of PriA K230R. Both PriA and PriA K230R complemented an extract of AT3327 *priA1::kan* to promote high levels of cointegrate formation (Figure 3(b), lanes 3 and 4), consistent with our observation that phage growth can occur, albeit at a reduced rate, when PriA is defective for helicase activity. These results indicate that other host proteins can carry out the function performed by PriA helicase. How well they do so *in vivo* may be influenced by cell growth conditions such as cell density.

Both PriA and PriA K230R can bind to the forked DNA structure created by Mu strand transfer

Although PriA is needed to initiate Mu DNA replication, the type of PAS sequences that are on φX174 DNA and pBR322 could not be found at the Mu ends. We searched for φX-type PAS within the mini-Mu element of donor substrate pGG215 (Surette *et al.*, 1987; Figure 4(a)), which is readily converted to a cointegrate in the reconstituted Mu transposition and replication system. Denatured DNA fragments were assayed for their ability to

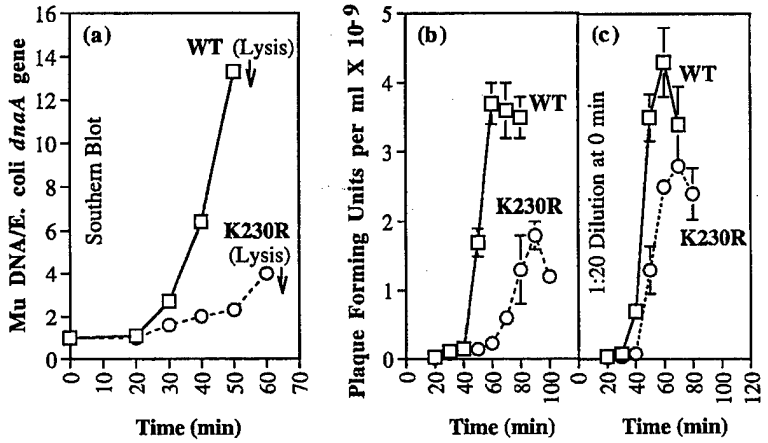


Figure 2. Deficiencies in Mu DNA replication supported by PriA K230R *in vivo*. (a) Mu DNA is amplified poorly during Mu lytic development in a PriA K230R strain (AT3853 *priA1::kan* pEL042 K230R) compared to a strain wild-type for PriA (AT3853 *priA1::kan* pEL042). A Southern blot of genomic DNA from samples collected at various points between induction (0 minute) and lysis was probed for Mu-specific and *E. coli*-specific (*dnaA* gene) sequences as described in Materials and Methods. The ratio of Mu signal/*E. coli dnaA* signal at 0 minute was set to 1. (b) and (c) Phage production is reduced in a PriA K230R strain but is improved by growth at lower cell density. Phage production was measured as described in Materials and Methods in cultures that were either left (b) undiluted or (c) diluted 20-fold at the point of induction (0 minute). Values are the average of three measurements with standard deviation of the mean shown by error bars.

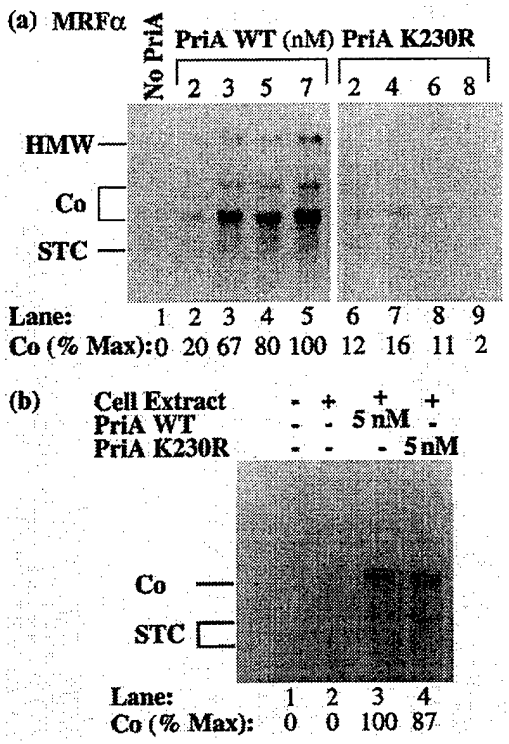


Figure 3. Differences between PriA and PriA K230R in their abilities to support Mu DNA replication *in vitro*. (a) PriA K230R lacks a function needed to initiate Mu DNA replication. Replication of STC1 in the reconstituted reaction system was conducted as described in Materials and Methods using PriA or PriA K230R as indicated. Autoradiographs of replication products are shown. In the most active reaction (lane 5) 90% of STC1 was converted to cointegrate (Co); this level was set to 100%. (b) The defective function of PriA K230R can be complemented by a crude cell extract (Fr II). Replication of STC1 supported by crude cell extract was conducted as described in Materials and Methods using PriA or PriA K230R as indicated. Autoradiographs of replication products linearized with *Eco*RI are shown. In the most active reaction (lane 3) >95% STC1 was converted to cointegrate; this level was set to 100%.

stimulate PriA's ATPase activity (Shlomai & Kornberg, 1980a,b; Zipursky & Marians, 1980), and the only PAS that were detected were the two present near the pBR322 origin (Figure 4(b), fragment D) located outside of the mini-Mu element. A donor substrate from which these two PAS were removed (pGG215ΔPAS) was active in Mu transposition and replication, using target DNA that also contains no PAS (data not shown). These results indicate that ϕX-type PAS are not required for Mu DNA replication.

PriA also binds to structures that resemble D-loops (McGlynn *et al.*, 1997), and this suggested that the PriA binding site may be created as the Mu ends are transferred to target DNA to form a branched DNA structure. In support of this hypothesis, band shift assays indicated that PriA binds to synthetic forked oligonucleotide substrates that mimic the DNA structure of the strand transfer product. A forked substrate containing the Mu right-end sequence was assembled from four oligonucleotides (Substrate A, Figure 5(a)). The duplex ahead of the fork consisted of 50 bp of Mu right end sequence with leading and lagging strand arms of 40 and 28 nt, respectively. As in the Mu strand transfer product, the fork is fully duplex except for a five-base gap between the leading strand primer and the fork. PriA was able to produce a discrete mobility shift with Substrate A (Figure 5(b), lanes 4-6), whereas it was unable to do so with the corresponding linear oligonucleotide that contains the Mu right-end (Substrate Z; Figure 5(b), lanes 1-3).

In addition, the deficiency of PriA K230R in promoting Mu DNA replication is not due to any defect in binding the Mu fork. PriA and PriA K230R bound to Substrate A (Figure 5(c)) with dissociation constants (K_D) of 21 and 19 nM, respectively, comparable to the K_D of 11 nM for binding PriA to the ϕX174 PAS (Ng & Marians, 1996a).

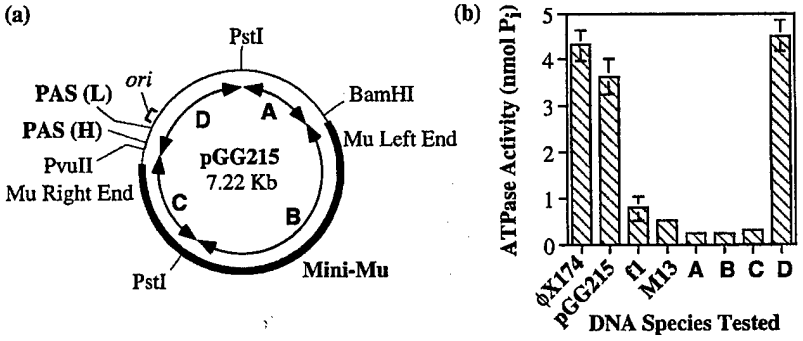


Figure 4. The Mu left and right ends do not contain PAS. (a) pGG215 donor substrate. The mini-Mu element is indicated in bold; the Mu left and right ends are on fragments B and C, respectively. The two pBR322 PAS (designated L and H; Zipursky & Marians, 1981) in the pGG215 vector are on fragment D. (b) Fragments that include regions of mini-Mu do not stimulate PriA's ATPase activity. Fragments A-D of pGG215 (subcloned into M13mp18) and full-length pGG215 were assayed for the ability to stimulate PriA's ATPase activity as described in Materials and Methods. Results are the average of three independent trials with standard deviation given by error bars.

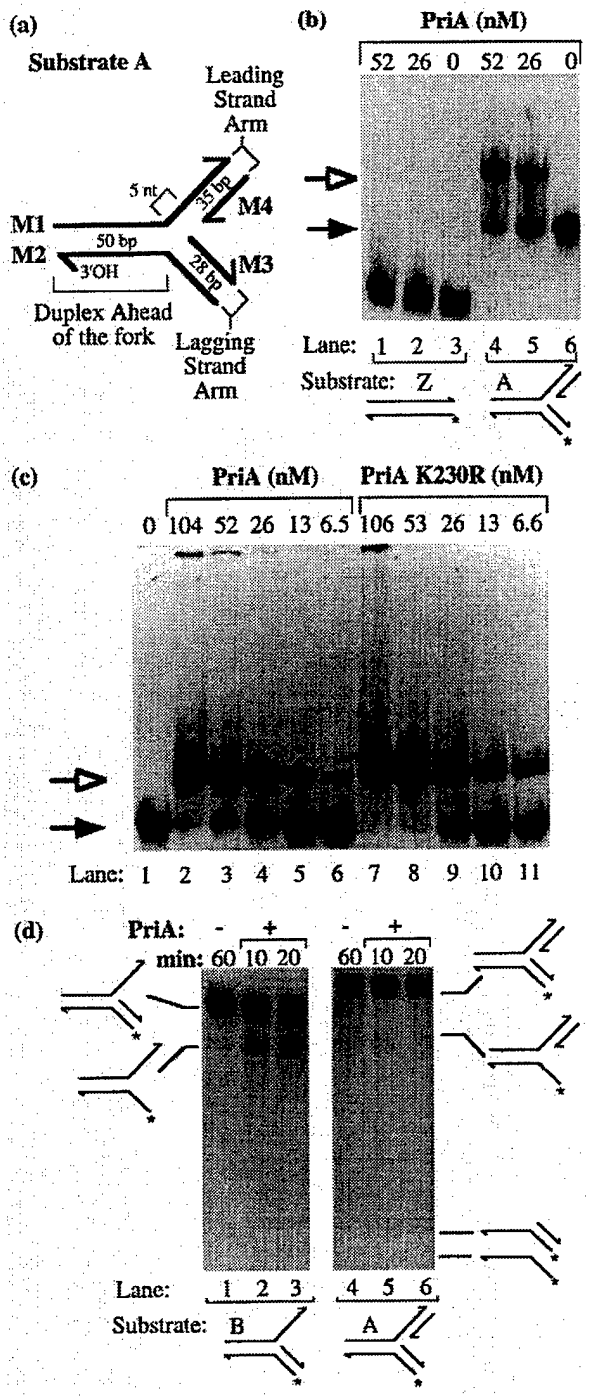


Figure 5. Ability of PriA to bind and unwind synthetic substrates resembling the Mu fork. (a) Substrate A (oligonucleotides M1, M2, M3, and M4). This substrate reflects the Mu fork DNA structure, with a five nucleotide (nt) opening present on the leading strand arm and a completely duplex lagging strand arm. (b) PriA binds to Substrate A but not to Substrate Z (oligonucleotides M2 and M5) in band shift assays. Band shifts were conducted as described in Materials and Methods. The filled arrow indicates the position of free Substrate A; the open arrow indicates the position of the shifted complex. (c) PriA and PriA K230R bind equally well to Substrate A in band shift assays. (d) PriA unwinds Substrate B (oligonucleotides M1, M2 and M3) more efficiently than Substrate A in helicase assays. Helicase assays that included PriA and SSB were conducted as described in Materials and Methods.

Unwinding of the lagging strand arm of a synthetic fork by PriA helicase

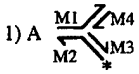
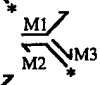
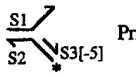
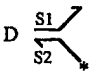
The role of PriA helicase in Mu DNA replication suggested that it may expose single-stranded DNA on the lagging strand side of a fork to load DnaB helicase. We investigated whether PriA could promote duplex opening at a fork as it promotes preprimosome assembly. Because PriA does not promote DNA synthesis as efficiently on the deproteinized strand transfer product as on the prereplisome STC3 (Jones & Nakai, 1997), naked DNA substrates with the exact structure of the Mu fork might not necessarily be the best substrates for PriA helicase. For this analysis we searched for suitable forked oligonucleotide structures that would serve as good substrates for PriA helicase but have insufficient single-stranded DNA to load DnaB onto the lagging strand template.

PriA helicase was more active on substrates with a single-stranded leading strand arm than on substrates with two duplex arms such as the Mu fork. Although PriA bound to both Substrate A (the Mu fork) and Substrate B (the analogous fork with single-stranded DNA on the leading strand arm) with nearly equal affinity (data not shown), its helicase was five times more active on Substrate B than on Substrate A (Figure 5(d), cf. lanes 1-3 with lanes 4-6; Table 1, lines 1 and 2). In the presence of SSB, the lagging strand arm (M2-M3 duplex) of Substrate B was unwound almost exclusively, most likely the result of PriA binding to the lagging strand template at the fork and translocating in a 3′ to 5′ direction.

A small gap may also facilitate access of PriA to the lagging strand template. PriA had little helicase activity on Substrate C (Table 1, line 3), a fork analogous to Substrate B but with a longer lagging strand arm and a different DNA sequence. Helicase action on Substrate C was comparable to that on Substrate B when a five-base gap was introduced on the lagging strand arm (Substrate C[− 5], Table 1, line 4). A gap of three nucleotides was sufficient to promote helicase action, whereas a gap of a single nucleotide was not (data not shown). PriA bound equally well to Substrates C and C[− 5] (data not shown), indicating that reduced helicase activity on Substrate C was not due to reduced binding affinity. Substrate C has a lagging strand arm (S2-S3 duplex) of 70 nt compared to 28 nt for Substrate B; in addition, it does not have Mu end sequences in the 30 bp duplex ahead of the fork (S1-S2 duplex). Although Substrate B has no single-stranded segment on the lagging strand template, some feature of Substrate B, such as the shorter length of the lagging strand arm or its DNA sequence, may permit exposure of single-stranded DNA on the lagging strand template, allowing PriA to initiate unwinding. The requirement of the gap in Substrate C for PriA helicase activity was not examined further at this time.

Our overall results indicated that Substrate C[− 5] was an ideal substrate for examining the

Table 1. PriA, DnaB, and preprimosome helicase activity on synthetic DNA fork substrates

Substrate	Proteins present	Total substrates consumed	Labeled products
1) A 	PriA, SSB	5%	3% 2%
2) B 	PriA, SSB	25%	**25%**
3) C 	PriA, SSB	3%	3%
4) C[-5] 	PriA, SSB	27%	**24%** 3%
5) C[-5]	PriA, PriB, PriC, DnaT, DnaBC, SSB	50%	**21%** 4% **25%**
6) C[-5]	PriA, PriB, PriC DnaT, SSB	43%	**39%** 1% 2%
7) C[-5]	PriB, PriC, DnaT, DnaBC, SSB	3%	2% 1%
8) C[-5]	PriA DnaBC, SSB	38%	**35%** 1% 2%
9) C[-5]	DnaBC, SSB	3%	3%
10) D 	DnaBC, SSB	31%	**31%**
11) D	PriA, PriB, PriC, DnaT, DnaBC, SSB	40%	**40%**

Helicase assays using the componenents indicated were performed as described in Materials and Methods. Oligonucleotide composition of each substrate is shown; the oligonucleotide designated with an asterisk is radiolabeled. Major products (>20% of total substrate) are highlighted in bold; any potential products not listed represent <1% total substrate. Less than 1% of substrate was consumed in control reactions including only SSB.

role of PriA helicase during preprimosome assembly. The gap of five nucleotides and the single-stranded leading strand arm allowed preferential PriA helicase action on the lagging strand arm in the presence of SSB (Table 1, line 4), but the gap is too small to provide a binding site for DnaB (Bujalowski & Jezewska, 1995; Table 1, line 9). If DnaB is bound to the lagging strand arm of Substrate C[− 5], its single-stranded leading strand arm would also allow efficient unwinding of the duplex ahead of the fork by DnaB. When DnaB unwinds DNA in the absence of DNA pol III holoenzyme, a 3′ single-stranded tail is required on the DNA strand to be displaced (LeBowitz & McMacken, 1986) even when DnaB is acting as part of the preprimosome (Lee & Marians, 1989).

Duplex opening by PriA can promote loading of DnaB onto the fork during preprimosome assembly

The preprimosome (PriA, PriB, PriC, DnaT, and DnaBC) in the presence of SSB efficiently unwound the duplex ahead of the fork on Substrate C[− 5] (S1-S2 duplex; 29% of the total substrate; Table 1, line 5). The major product of this process was single-stranded S2 (25% of total substrate), a result of unwinding both the S1-S2 and S2-S3 duplexes. When DnaBC was omitted from the reaction mixture (Table 1, line 6), very little of the S1-S2 duplex was unwound, although the S2-S3 duplex was still unwound at high levels. Very little of the substrate was unwound at all if PriA was omitted from the reaction mixture (Table 1, line 7). These results are consistent with a mechanism in which PriA unwinds the lagging strand arm to promote unwinding of the duplex ahead of the fork by DnaB.

Preprimosome assembly was required for unwinding the S1-S2 duplex in this reaction. When PriB, PriC and DnaT were omitted, only the lagging strand arm could be unwound at high levels (Table 1, line 8). PriB, PriC, and DnaT bring PriA and DnaB together in a single complex (Liu *et al.*, 1996; Ng & Marians, 1996a,b). In contrast, PriABC and DnaT were not required for DnaB helicase action if the lagging strand arm of the fork was single-stranded. Substrate D, which has two single-

stranded arms, was unwound in the presence of DnaBC without the remaining preprimosome components (Table 1, cf. lines 10 and 11), provided that DnaB was allowed to bind to the substrate before the addition of SSB (data not shown).

We confirmed that PriA helicase activity was essential for unwinding of the S1-S2 duplex of Substrate C[− 5] by the preprimosome; little or no unwinding could be detected when PriA K230R replaced PriA (Figure 6(a)). In addition, we were able to distinguish participation of the two helicases by taking advantage of their different nucleotide requirements when ATP is not the major energy source (Lasken & Kornberg, 1988; LeBowitz & McMacken, 1986; Lee & Marians, 1987, 1989). Unwinding of the S1-S2 duplex of Substrate C[− 5] by the preprimosome components required both dATP and GTP to support the PriA and DnaB helicases, respectively, as well as low levels of ATP

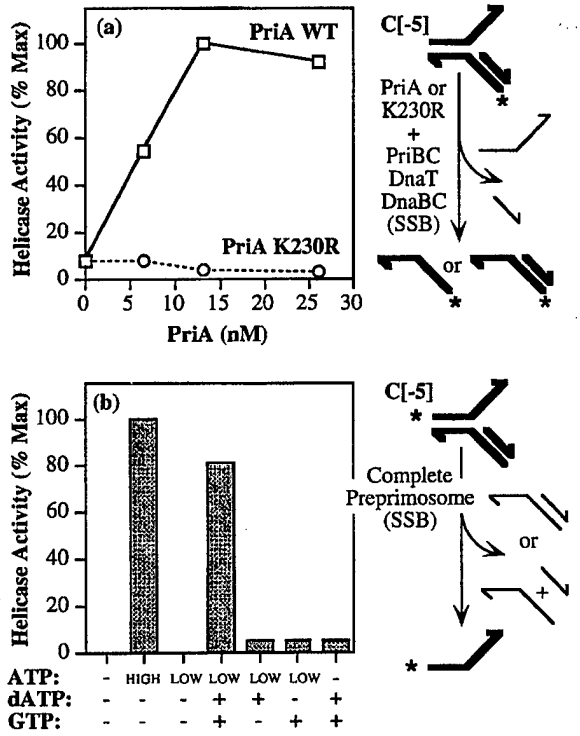


Figure 6. Contribution of the two preprimosomal helicases in unwinding forked substrates. (a) PriA K230R does not support unwinding of the S1-S2 duplex on Substrate C[− 5] by the preprimosome. Helicase assays that included PriA or PriA K230R as well as PriB, PriC, DnaT, DnaBC and SSB were performed as described in Materials and Methods. Total unwinding of the S1-S2 duplex was measured. The level in the most active reaction (13 nM PriA WT) was set to 100%. (b) Nucleotide requirements for unwinding of the S1-S2 duplex in Substrate C[− 5] by the preprimosome. Helicase assays that included PriA, PriB, PriC, DnaT, DnaBC and SSB were supplemented with ATP (2 mM [HIGH] or 10 μM [LOW]), dATP (2 mM) and GTP (3.4 mM) as indicated. Production of S2 in the most active reaction (2 mM ATP) was set to 100%. Results are the average of two independent trials; duplicate values varied by less than 5%.

(≤10 μM; Figure 6(b)). By itself this ATP concentration is insufficient to fuel S1-S2 duplex unwinding on Substrate C[− 5] (Figure 6(b)), but it probably plays a role in assembly or activation of the preprimosome to elicit its two helicase activities (Lee & Marians, 1989). In the absence of dATP needed to drive PriA helicase action, unwinding of the substrate was very low (Figure 6(b)), consistent with the inability of PriA K230R to promote unwinding of the S1-S2 duplex. In the absence of GTP, the S2-S3 duplex was unwound (data not shown), confirming that PriA could be driven by dATP, but only very low levels of S1-S2 duplex unwinding were observed (Figure 6(b)). In contrast, unwinding of Substrate D, which has two single-stranded arms, could be fueled by GTP alone (data not shown), consistent with the ability of DnaB to unwind this fork. These results demonstrate that both the 3′ to 5′ helicase of PriA and the 5′ to 3′ helicase of DnaB are needed to unwind the S1-S2 duplex on Substrate C[− 5] and that this process requires preprimosome assembly to promote the concerted action of the two helicases. This strongly suggests that unwinding of the S2-S3 duplex and the loading of DnaB onto the fork are coupled events.

Duplex opening by PriA is coupled to loading of DnaB during preprimosome assembly

We confirmed that unwinding of the S2-S3 and S1-S2 duplexes of Substrate C[− 5] by the preprimosome is tightly coupled using a competition experiment. The preprimosome components were first allowed to associate with this substrate at 0 °C, conditions that do not allow any helicase action (data not shown). Even after subsequent challenge with 100-fold excess competitor DNA (the single-stranded oligonucleotide M5), both duplexes were still unwound to form the single-stranded S2 product (Figure 7(a), open triangles). This level of competitor effectively inhibited action of PriA or the preprimosomal helicases if these proteins were not first allowed to associate with the substrate (Figure 7(a) and (b), filled symbols). Moreover, the DnaB helicase activity on Substrate D, which could be produced from Substrate C[− 5] by the action of PriA, was effectively inhibited by the competitor even when the substrate was first incubated together with DnaBC (Figure 7(c)). These results indicate that when the preprimosome components unwind the S2-S3 duplex of Substrate C[− 5], they promote binding of DnaB before running off the template, coupling the processes of duplex opening and preprimosome assembly.

Discussion

Role of PriA helicase in duplex opening and replisome assembly at the Mu fork

A universal step in the initiation of DNA replication is the opening of the DNA duplex to promote

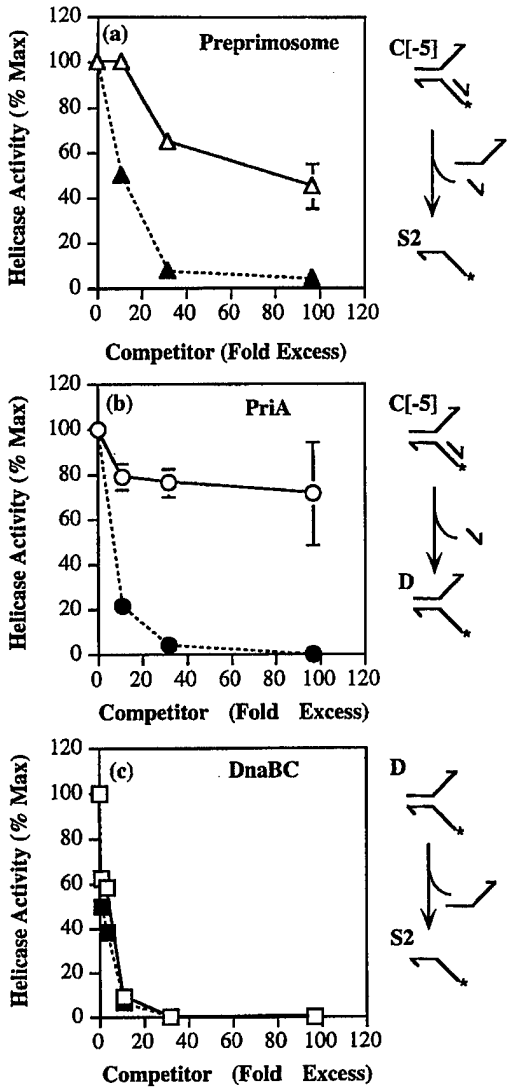


Figure 7. Duplex opening by PriA is tightly coupled to loading of DnaB onto forked substrates. Preprimosome components were initially incubated with substrate for ten minutes on ice before the addition of competitor DNA (the single-stranded oligonucleotide M5) and SSB followed by a 15 minute incubation at 30 °C (open symbols); alternatively, competitor DNA was present during the ten minute incubation on ice (filled symbols). Values are the average of multiple independent trials with standard deviation of the mean provided by error bars. (a) The preprimosome unwinds the two duplexes of Substrate C[−5] in the presence of excess competitor. Helicase assays included preprimosomal components PriA, PriB, PriC, DnaT, and DnaBC. Production of S2 in the absence of competitor was set to 100 %. (b) PriA unwinds the S2-S3 duplex of Substrate C[−5] in the presence of excess competitor. Helicase assays included the preprimosomal component PriA. The accumulation of Substrate D product was quantitated. Production of Substrate D in the absence of competitor was set to 100 %. (c) Helicase activity of DnaB on Substrate D is inhibited by challenge with competitor. Helicase assays included the preprimosomal component DnaBC complex. Production of S2 in the absence of competitor was set to 100 %.

binding of the major helicase DnaB, a process that ultimately leads to the assembly of the replisome. For bacterial chromosomal replication the DnaA protein serves the function of opening the duplex (Bramhill & Kornberg, 1988) and recruiting DnaB helicase to form the prepriming complex at *oriC* (Baker *et al.*, 1986; Funnell *et al.*, 1987). For initiation of Mu DNA replication by transposition, assembly proteins PriA, PriB, PriC, and DnaT of the φX-type primosome are involved in recruiting DnaB to the initiation site (Jones & Nakai, 1997). On synthetic DNA forks that have insufficient single-stranded DNA on the lagging strand arm to bind DnaB (as is the case with the Mu fork), the PriA helicase unwinds this duplex arm while promoting preprimosome assembly and binding of DnaB to DNA. Mu DNA replication *in vivo* proceeds at less than optimal rates when the PriA helicase is inactive, and PriA helicase is required for significant levels of Mu DNA replication *in vitro* in the reconstituted system, indicating that the PriA helicase can catalyze a critical step in initiation of Mu DNA replication. These results are consistent with a mechanism where PriA opens the duplex at the Mu fork to create a binding site for DnaB.

Our results indicate that during preprimosome assembly at a fork, DnaB binds to the same strand as PriA. The bidirectional helicase activity of the preprimosome was first demonstrated by assembling the complex on the phage φX174 PAS (Lee & Marians, 1989). However, the opposing helicase activities of PriA and DnaB raises the possibility that PriA at a replication fork might normally translocate 3′ to 5′ along the leading strand template, augmenting DnaB's progress on the lagging strand template. The role of PriA helicase at the Mu fork illustrates the utility of coupling two helicases moving in opposite directions on the same strand.

It is not yet clear how PriA would initiate helicase action on the Mu strand transfer product. Forked oligonucleotide substrates with the structure of the Mu fork are generally not good substrates for PriA helicase (e.g. Table 1, line 1). This is consistent with the observation that the preprimosome and DNA pol III holoenzyme initiate DNA replication less efficiently on the deproteinized strand transfer product than on the prereplisome STC3 (Jones & Nakai, 1997). There is also the problem of directing PriA to translocate along the lagging strand template rather than the leading strand template so that DnaB is loaded onto the correct strand. One possibility is that the prereplisome proteins ($MRF\alpha_2$) in STC3 promote PriA helicase action on the lagging strand arm of the fork. After completion of strand transfer, the transpososome is displaced by the prereplisome proteins with the aid of the molecular chaperone ClpX (Kruklitis & Nakai, 1994; Kruklitis *et al.*, 1996), and these proteins allow Mu DNA replication to proceed only by a PriA-dependent pathway.

In our current model for initiation at the Mu fork (Figure 8), PriA plays the function analogous

to that of DnaA at *oriC* by recognizing the initiation site created by strand transfer and opening the duplex for replisome assembly. We speculate that the prereplisome proteins present in STC3 (Figure 8(a)), while not required for PriA binding, may direct PriA to the lagging strand arm of the Mu fork. The binding of PriA to the Mu fork (Figure 8(b)) promotes recruitment of the other preprimosome proteins (Figure 8(c) and (d)). Initiation of PriA helicase action unwinds the lagging strand arm of the fork (Figure 8(d)), and once enough single-stranded DNA is exposed, DnaC dissociates from DnaB allowing DnaB to bind to the DNA (Funnell *et al.*, 1987; Learn *et al.*, 1997; Wahle *et al.*, 1989a,b). PriA and DnaB may then translocate in opposite directions on the lagging strand template (Figure 8(e)). However, the 3′ to 5′ helicase activity of the preprimosome requires significantly higher NTP concentrations than the 5′ to 3′ helicase activity (Lee & Marians, 1989), a property that may eventually cause PriA to disengage from the lagging strand template. Once DnaB is bound to the lagging strand template, DNA pol III holoenzyme can then assemble at the fork through its interaction with the primer-template and with DnaB (Yuzhakov *et al.*, 1996; Figure 8(f)), completing the assembly of the replisome. If PriA is defective in helicase activity, other helicases or a 5′ to 3′ nuclease could create a single-stranded segment on the lagging strand template for DnaB loading. But in such a mechanism, the process of duplex opening and DnaB loading would not be so tightly coupled, and the rate at which DNA replication is initiated may be relatively slow.

General function of the preprimosome and its two helicases in the replication of the host chromosome

A major question regarding the PriA helicase has been its function in cellular DNA replication and recombination and its relationship to PriA's role in primosome assembly. Knock-out mutations of the *priA* gene are not lethal but have serious consequences including slow growth, poor viability, sensitivity to DNA damaging agents, and characteristics of a constantly induced SOS response (Lee & Kornberg, 1991; Nurse *et al.*, 1991).

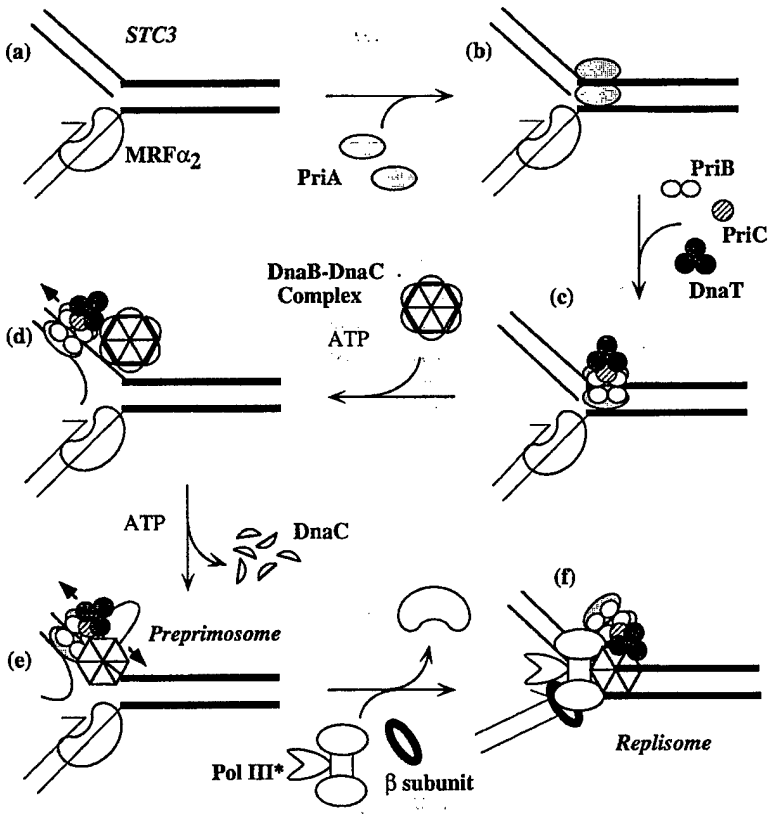


Figure 8. Model for PriA helicase-assisted assembly of the replisome during Mu transposition. (a) The prereplisome STC3 includes host protein components ($MRF\alpha_2$) which protect the leading strand primer. (b) PriA binds to the lagging strand template at the fork. (c) PriB, PriC and DnaT enter the PriA-DNA complex. (d) The DnaB-DnaC complex associates with the PriABC-DnaT complex, and the 3′ to 5′ helicase of PriA unwinds the lagging strand arm to create a binding site for DnaB. (e) With the exit of DnaC from the complex, DnaB is loaded onto the lagging strand completing assembly of the preprimosome. The opposing 3′ to 5′ and 5′ to 3′ helicase activities of the preprimosome could form a single-stranded loop on the template. (f) The association of DNA pol III holoenzyme with the leading strand primer-template and DnaB completes assembly of the replisome, with the hypothetical exit of $MRF\alpha_2$. PriA may eventually dissociate from the lagging strand template to terminate action of the 3′ to 5′ helicase.

Expression of PriA that is defective in helicase activity can restore the wild-type phenotype in essentially all respects (Zavitz & Marians, 1992), and helicase-deficient PriA proteins such as PriA K230R are fully active in promoting primosome assembly on the ϕX174 template (Zavitz & Marians, 1992). These data suggest that the primosome assembly function, but not the helicase function, plays a critical role in the replication and maintenance of the chromosome. Nevertheless, helicase mutants do not necessarily restore full transformation efficiency of pBR322-based plasmids (Zavitz & Marians, 1992) or full efficiency in inheritance of genetic markers by P1 transduction (Sandler *et al.*, 1996). PriA helicase could accelerate the rate at which DnaB is loaded by expanding the duplex opening when insufficient single-stranded DNA is available.

It has been suggested that PriA together with proteins that promote homologous recombination may function in reassembly of the replisome when a replication fork stalls at a lesion or interruption in the template (Asai *et al.*, 1994; Bierne & Michel, 1994; Courcelle *et al.*, 1997; Kogoma, 1997; Kuzminov, 1995; Nurse *et al.*, 1991; Rupp & Howard-Flanders, 1968; Zavitz & Marians, 1992). We have found that the preprimosome readily assembles on a forked substrate with a single-stranded leading strand arm, and such a substrate could result if DNA polymerase encounters a blockage on the leading strand template. If lagging strand synthesis continues uncoupled from leading strand synthesis, a single-stranded gap on the leading strand template would be created. Formation of such a product has been observed when DNA replication was reconstituted with eukaryotic cell extract on templates that have thymine dimers (Svoboda & Vos, 1995). Such single-stranded gaps created by DNA replication are thought to provide the SOS-inducing signal in *Escherichia coli* (Sassanfar & Roberts, 1990). On the resulting stalled fork, there may not be sufficient single-stranded DNA available on the lagging strand arm to allow restart of replication. While a nuclease or other helicase could potentially expose a region of single-stranded DNA, the most efficient method of creating the duplex opening is to couple PriA helicase action with preprimosome assembly.

The ability of PriA to bind to forked structures, open the duplex and promote primosome and replisome assembly is similar to the function carried out by the initiator DnaA at *oriC*. The major difference is that the signal to initiate replication for PriA is the DNA structure found at stalled forks and recombination intermediates. Once assembled, the protein composition of the preprimosome is conserved as the replisome translocates along the DNA template (Ng & Marians, 1996b). PriA in the replisome may facilitate restart of replication if the replisome encounters lesions or breaks in the DNA. PriA's ability to translocate in a direction opposite to DnaB may promote reopening of the duplex to reassemble the replisome as well as prevent disassembly of the preprimosome by allowing it to back off from the DNA lesion. While it is the task of DnaA to coordinate chromosomal replication with the cell cycle, the fully functional ϕX-type primosome would act as a mobile initiator that helps keep interruptions in the progression of the replication fork to a minimum.

Materials and Methods

Plasmids and bacterial strains

pND706-PriA was a gift from Nick Dixon (Australian National University); pND706-PriA K230R (described below) was used to overproduce PriA K230R. pEL042 (Lee *et al.*, 1990) was a gift from Elliott Crooke (Georgetown University); this plasmid expresses PriA from its own promoter. To construct pND706-PriA K230R and pEL042 K230R, a single base substitution (A to G) at position 752 in the *priA* gene (Lee *et al.*, 1990) was introduced into both plasmids using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene®) according to manufacturer's instructions. The mutagenized plasmids were sequenced by Veritas, Inc. (Rockville MD).

AT3327 and AT3327 *priA1::kan* have been described (Jones & Nakai, 1997). To construct AT3853 *priA1::kan*, the *priA1::kan* mutation (Lee & Kornberg, 1991) was introduced into AT3853 (Mucts62) by P1 transduction. Both *priA1::kan* strains were maintained on minimal media (Masai *et al.*, 1994) supplemented with 0.2% (w/v) Casamino acids and 25 μg/ml kanamycin. Following $CaCl_2$ transformation (Sambrook *et al.*, 1989) with pEL042 or pEL042 K230R, strains were routinely grown on LB (Sambrook *et al.*, 1989) supplemented with 50 μg/ml ampicillin.

Mu growth *in vivo*

Plating efficiency on AT3327 *priA1::kan* transformed with either pEL042 or pEL042 K230R was determined as described (Jones & Nakai, 1997); results represent four independent trials. To examine the kinetics of phage production and Mu DNA amplification, 200 ml cultures of AT3853 *priA1::kan* transformed with either pEL042 or pEL042 K230R were grown to an A_{600} of 0.4 (1.5×10^8 cells/ml) at 30 °C, adjusted to a final concentration of 5 mM $MgSO_4$ and 0.2% (w/v) glucose, and then induced at 42 °C for 90 minutes. In some cases cultures were diluted 20-fold at the start of induction (0 minute). Phage production at various times postinduction was measured by plating dilutions of the cultures in duplicate with indicator bacteria (AT3327). Alternatively, genomic DNA from sampled cultures was subjected to Southern blot analysis, performed and quantitated as described (Jones & Nakai, 1997).

Proteins

All restriction enzymes, DNA pol I, *E. coli* DNA ligase, and T4 polynucleotide kinase were purchased from New England BioLabs. Purification of PriA K230R was essentially as described for PriA by Marians (1995) with the exception that a Sephacryl® S-200 HR HiPrep® 16/60 column (Amersham Pharmacia Biotech) was used for preparation of Fr IV. Concentrations of PriA and PriA K230R were determined by the method described by Pace *et al.* (1995). All other proteins, crude cell extract

(Fr II), and MRFα (Fr III) were prepared as described (Jones & Nakai, 1997).

Reconstituted Mu DNA replication assay

Replication of STC1 (50 fmol as complex) was carried out with 55 fmol DNA pol III*, 190 fmol (as monomer) DNA pol III β subunit, 1.2 pmol DnaG (as monomer), 130 fmol DnaBC complex ($DnaB_6$-6DnaC), 15 pmol SSB (as tetramer), 900 fmol gyrase (as gyrA-gyrB dimer), 8.4 pmol ClpX (as monomer), 900 fmol ClpP (as tetradecamer), 0.01 unit of DNA pol I, 1 unit *E. coli* DNA Ligase, 60 fmol PriB (as dimer), 130 fmol PriC (as monomer), 470 fmol DnaT (as trimer), PriA or PriA K230R as indicated, and crude cell extract (12 units) or MRFα (12 units) in a 50 μl reaction mixture as described (Jones & Nakai, 1997). Replication products were deproteinized and separated on 0.6% agarose gels in alkaline electrophoresis buffer (Sambrook *et al.*, 1989). Gels were neutralized and stained with 0.5 μg/ml ethidium bromide and then dried and subjected to phosphorimagery and autoradiography.

PriA ATPase assay

Fragments of pGG215 (Figure 4(a)) were subcloned into M13mp18 (Gibco BRL Life Technologies™), and replicative form (RF) DNA from these clones as well as φX174 RF, M13mp18 RF, f1 RF and pGG215 were used in PriA ATPase assays. Linear, double-stranded DNA was heated to 100 °C for ten minutes, then quickly cooled in an ice water-bath for five minutes prior to addition to the assay. The assay (15 μl total volume) was conducted in 50 mM Hepes-KOH (pH 8.0), 10 mM MgOAC, 1 mM DTT, 100 mM potassium glutamate, 0.1 mg/ml bovine serum albumin, 10 μg/ml rifampicin, 0.7 mM [γ-^{32}P]ATP (DuPont NEN®) plus 2 fmol (as duplex linear molecule) DNA, 170 fmol PriA (as monomer) and 15 pmol SSB (as tetramer). Reactions were incubated for 60 minutes at 37 °C, then stopped by the addition of 3 μl of 200 mM EDTA. A portion of each reaction (3 μl) was spotted on a PEI-cellulose thin layer chromatography plate (J.T. Baker) which was developed in 0.5 M LiCl, 4.6% (v/v) formic acid, dried and subjected to phosphorimagery. Generation of free ^{32}P phosphate was quantitated.

DNA substrates for band shift and helicase assays

Synthetic DNA substrates were constructed from the following oligonucleotides (Gibco BRL Life Technologies™): S1-CCATTAGCAAGGCCGGAAACGTCACC AATGCAACGATCAGCCAACTAAACTAGGACATCT-TTGCCCACCA; S2-CGCTACAGTCTGACGCTAA-AGGCAAACTTGATTCTGTCGCTACTGATTACGG TGCTGCTATCGATGGTTTCATTGGTGACGTTTC-CGGCCTTGCTAATGG; S3-AAACCATCGATAG-CAGCACCGTAATCAGTAGCGACAGAATCAAG-TTTGCCTTTAGCGTCAGACTGTAGCG; S3[-5]-ATCGATAGCAGCACCGTAATCAGTAGCGACA-GAATCAAGTTTGCCTTTAGCGTCAGACTGTAGCG; M1-GTTTTCGCATTTATCGTGAAACGCTTTCGCG-TTTTTCGTGCGCCGCTTCATGTACACCGTTCATCT-GTCCTCGTTCAAAGTTGGTCAGTT; M2-AAGCT-GTGGTGGTAACAAGTAGTGCCGGTGAAGCG GCGC-ACGAAAAACGCGAAAGCGTTTCACGATAAATGC-GAAAAC; M3-CCGGCACTACTTGTTACCACCA-CAGCTT; M4-AACTGACCAACTTTGAACGAGGAC-AGATGAACGGT; M5-GTTTTCGCATTTATCGT-GAAACGCTTTCGCGTTTTTCGTGCGCCGCTTCAC-CGGCACTACTTGTTACCACCACAGCTT. The oligonucleotide composition of each substrate is provided in appropriate Figure legends and Table 1. For each substrate, one oligonucleotide was radiolabeled with ^{32}P to a specific activity of 2×10^6 to 5×10^6 CPM/pmol using T4 polynucleotide kinase. This oligonucleotide (10 pmol) was combined with two- to fourfold excess of various unlabeled oligonucleotides in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 M NaCl, and the mixture was heated to 90 °C then slowly cooled to 40 °C. Annealed complexes were separated on 10% polyacrylamide gels (cross-linked at a ratio of 30:1) in TBE buffer (Sambrook *et al.*, 1989), and purified using the Elutrap® system (Schleicher & Schuell). The oligonucleotide composition of various substrates was confirmed by labeling all oligonucleotides in the purified substrate and separating them on a denaturing polyacrylamide gel.

Band shift assay

Band shifts were conducted essentially as described by McGlynn *et al.* (1997) using DNA substrates (16 fmol) and PriA or PriA K230R (0.13-1.1 pmol as monomer) in 20 μl reaction mixtures. Band shift gels were dried and subjected to phosphorimagery and autoradiography. The K_D value was determined as described by Ausubel *et al.* (1992) using the data shown in Figure 5(c).

Helicase assay

DNA substrates (16 fmol) were combined in 20 mM Tris-HCl (pH 7.5), 5.4 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml bovine serum albumin, and 2 mM ATP unless otherwise indicated (20 μl total volume) with the following proteins as indicated: 260 fmol PriA or PriA K230R (as monomer), 60 fmol PriB (as dimer), 2.2 pmol PriC (as monomer), 2.4 pmol DnaT (as trimer), 500 fmol DnaBC complex, and 240 fmol SSB (as tetramer). Reaction mixtures excluding SSB were incubated on ice for ten minutes. SSB was then added and reactions were incubated for 15 minutes (unless otherwise indicated) at 30 °C. Deproteinized products were separated on 10% polyacrylamide gels (cross-linked at a ratio of 30:1) in TBE buffer (Sambrook *et al.*, 1989) at 140 V for 2.5 hours. Gels were dried and subjected to phosphorimagery and autoradiography. All experiments included a negative control, a reaction mixture to which only SSB was added (e.g. Figure 5(d), lane 1), and markers representing potential helicase products. The percent of total substrate converted to each product was calculated.

Other

All quantitation was by phosphorimagery using the Molecular Dynamics Storm 840 system and ImageQuant™ 1.11 B15 software.

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